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VOLUME 84 NUMBER 3

# BULLETIN of the JOHNS HOPKINS HOSPITAL

THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL
SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY

## March, 1949

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PUBLISHED MONTHLY

THE JOHNS HOPKINS PRESS, Baltimore-18, Maryland

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*This periodical is issued monthly.* Two volumes a year are issued, each consisting of approximately 400 pages, and beginning in January and July, respectively.

*Subscription price.* In the United States and its possessions $6.00 per year. In Canada $6.25 per year; in foreign countries $6.50 per year. Prices for back volumes and back single numbers on application. Single numbers of the current volume, when available, $.75. Prices are net, postpaid.

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THE JOHNS HOPKINS PRESS
BALTIMORE-18, MARYLAND, U. S. A.

Entered as second-class matter at the Baltimore, Md., Post Office. Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917. Authorized on July 3, 1918.

# TISSUE CULTURE STUDIES ON LIVER CELLS OF ANAPHYLACTICALLY (ARTHUS) SENSITIZED ANIMALS IN THE PRESENCE OF THE SENSITIZING ANTIGEN

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Received for publication July 8, 1948

A difference between the anaphylactic type of hypersensitivity and the tuberculin type has been clearly demonstrated in tissue cultures of fibroblasts, polymorphonuclear leucocytes, lymphocytes, macrophages, and young marrow cells, by Rich and Lewis (1) and Aronson (2, 3). This difference consists in the fact that the washed cells of these types from an animal showing the tuberculin type of hypersensitivity are injured and killed by contact with the specific antigen in tissue cultures, whereas cells of these types from an animal sensitized anaphylactically to the degree of reacting with a necrotizing Arthus phenomenon are not visibly affected by *in vitro* contact with the specific antigen. These results are unaffected by the presence or absence of antibody in the culture medium. Furthermore, Rich and Follis (4) showed that to elicit the Arthus phenomenon in the cornea of a rabbit the cornea first has to be made vascular. They came to the conclusion that in the anaphylactic Arthus phenomenon there is vascular sensitivity, but the cells of the tissues at large are not sensitized; and that the tissue death in the Arthus reaction results primarily from impairment of nutrition by vascular damage.

It is known that focal necroses of the liver may occur when the specific antigen is injected into the circulation of the anaphylactically sensitized animal under conditions which permit the survival of the animal. Longcope (5) suggested that these focal necroses of liver cells might be due to a toxic substance liberated either into the blood stream, or inside the liver cells, during the anaphylactic reaction. Apitz (6) thought that the necroses might be due to passive congestion, but since they are often periportal rather than central there would seem to be little support for that view. Hartley and Lushbaugh (7), in more recent studies, concluded that the focal necrosis that occurs

under these conditions is due to contact of the antigen with antibody in or on the liver cells and is not dependent upon vascular damage. Since the question of the sensitivtiy of epithelial cells of the anaphylactically sensitized body has not been previously examined by *in vitro* studies (which are the only means suitable for determining whether the injurious effects of the specific antigen are due to a direct action upon the cells in question), the present *in vitro* study of the effect of antigen upon the liver cells of anaphylactically sensitized animals was conducted.

## EXPERIMENTAL METHODS

Adult male albino rabbits, weighing about 2500 gms., were used. They were kept in separate cages and were supplied daily with an abundance of Purina Chow and water.

Sterile horse serum, without preservative, was used as the antigen. The serum was injected subcutaneously into the rabbit for several weeks, in amounts of 1 cc. every three days. Skin tests for hypersensitivity were made at intervals by the injection of 0.1 cc. of horse serum into the skin of the flank. Sensitization was continued until the test injection produced an Arthus phenomenon with necrosis. Ten days after this degree of hypersensitivity had been attained, a piece of liver was removed by biopsy under aseptic precautions, using ether anaesthesia. This biopsy was used for tissue culture. Immediately after the operation the skin test was repeated. Only when necrosis recurred on this re-test were the results of the culture of the liver tissue considered significant. For every experiment in which liver from an Arthus sensitized rabbit was cultured, a control experiment was carried out, in which liver from a normal rabbit was used.

## TISSUE-CULTURE METHODS

Blood was obtained from the normal and the hypersensitive rabbits aseptically by cardiac puncture. Graded amounts of Lederle's synthetic heparin were added to 10 cc. portions of blood, in order to obtain satisfactory plasma for use in cultures. A dilution of 1–50,000 proved most satisfactory. Serum was obtained from the remainder of the blood.

The media used were made up in two parts. The first (Part 1) con-

sisted of one part beef embryo extract, one part balanced salt solution, Gey (8), and four parts of serum, rabbit serum alone or with horse serum in proportions given in Table I. The second (Part 2) consisted of one part chicken plasma and three parts rabbit plasma. In each experiment a piece of liver removed at biopsy was minced, under aseptic precautions, with detachable knife blades[1] into pieces approximately 0.5–1.0 mm. in diameter. Four of these small pieces of liver were

TABLE I

*Outline of typical experiment*

| NO CULTURES | WASHED LIVER CELLS FROM | PLASMA + SERUM FROM | PERCENT OF HORSE SERUM IN MEDIUM | NO. OF CULTURES SHOWING GOOD GROWTH AND VIABILITY OF LIVER CELLS |
|---|---|---|---|---|
| 35 | Arthus Rabbit | Arthus Rabbit | 0 | 30 |
| 35 | " " | " " | 30% | 31 |
| 35 | " " | " " | 10% | 23 |
| 35 | " " | Normal Rabbit | 0 | 21 |
| 35 | Normal Rabbit | Arthus Rabbit | 0 | 31 |
| 35 | " " | " " | 30% | 30 |
| 35 | " " | " " | 10% | 28 |
| 35 | " " | Normal Rabbit | 0 | 19 |
| 10 | Arthus Rabbit | Arthus Rabbit | 1:100–100% | 8 |
| 10 | " " | " " | 1:1,000 | 9 |
| 10 | " " | " " | 1:10,000 | 8 |
| 10 | Normal Rabbit | " " | 1:1,000 | 8 |

placed on a sterile cover slip (30 x 35 mm.), and three drops of Part 1 were added, followed by two drops of Part 2. Clotting resulted promptly after the addition of Part 2. The cover slip was then inverted and sealed with vaseline to a metal ring attached to a slide with vaseline. The cultures were then placed in an incubator, maintained at 37°C., and were examined daily. Generally at the end of four to six days the cultures were fixed in Vandergrift's solution (9) for five minutes. They were then allowed to stand overnight in 1% ammonia alcohol (95%), then they were washed, stained in dilute Giemsa (20 drops to 50 cc. of water) for 24 hours, differentiated in 95% alcohol and dehydrated in acetone and xylene.

[1] Bard Parker, size 11.

In five experiments four types of media were used, in order that the cells of the sensitized and of the control animals could be studied in the presence or absence of the specific antigen, and in the presence or absence of antibody-containing rabbit plasma. One hundred and forty cultures, each containing four explants per culture, were made from the liver of each animal. Thus there were 560 explants of the liver of each animal in each of these experiments. In one experiment in which greater dilutions of the antigen were used only 40 cultures were made (Table I).

## RESULTS

The growth of liver cells extended in sheets into the zone around the explants on about the third day. These cells were large, with abundant, pale staining cytoplasm, the margins of which were quite sharply outlined. Their nuclei were large, oval or round, rather vesicular, had a very sharply defined limiting membrane and generally contained one large nucleolus. The cells as well as the nuclei varied considerably in size, some being two to four times larger than others. To differentiate the growth of bile duct epithelium from that of liver cells, several cultures of the epithelium of the common bile duct were made. The latter cells were different in appearance from the liver cells. They grew out in sheets, but the individual cells were smaller and more regular in size, and their nuclei were also much smaller than those of the liver cells (Figures 1 and 2).

Microscopical study of the growing and stained cultures did not show any qualitative or quantitative difference between the growth or appearance or duration of viability of the liver cells of normal rabbits growing in the presence of horse serum, and those of the hypersensitive rabbits growing in the presence of horse serum, whether in the presence or absence of antibody. If anything, both the normal and the hypersensitive cells grew somewhat better in the culture medium that contained horse serum than in the medium without horse serum (Figures 3 and 4).

## DISCUSSION

In the present experiment, animals were sensitized anaphylactically to the degree that they responded with a necrotizing Arthus phenom-

enon to the intracutaneous injection of 0.1 cc. of the antigen, at which time their liver cells were exposed to contact with the specific antigen in tissue cultures. The results show that, even in so highly sensitized an animal, the epithelial liver cells, as has been previously shown for macrophages, polymorphonuclear leucocytes, lymphocytes, fibroblasts and young marrow cells, are not damaged *in vitro* by direct contact with the specific antigen. This is in marked contrast to the state of affairs in tuberculin type hypersensitivity, in which, as shown by Rich and Lewis (1) and others, the cells so far tested (polymorphonuclear leucocytes, lymphocytes, fibroblasts and immature marrow cells) are killed by contact with the specific antigen *in vitro*. Indeed, Moen (10) has shown that the cells from animals with tuberculin type hypersensitivity retained their hypersensitivity for numerous generations in tissue culture.

The liver cells from the sensitized animals in the present experiments were exposed to concentrations of the specific antigen ranging between 1–3 and 1–10,000, in the presence and in the absence of specific antibody. In no case was any specific inhibition of growth or viability detectable. The present experiments, therefore, offer no support for the view, suggested by others, that the epithelial hepatic cells of the Arthus sensitized body are, themselves, sensitized in a manner that would cause them to be injured by direct contact with the specific antigen. The epithelial hepatic cells of the Arthus sensitized body thus correspond with the fibroblasts, macrophages, polymorphonuclear leucocytes, lymphocytes, and immature marrow cells in being unaffected by contact with the specific antigen *in vitro*. This would suggest that the focal necroses that occur in the liver of the Arthus sensitized animal, following the intravascular injection of the antigen, result from a vascular alteration, just as the available evidence, reviewed above, indicates that the necrosis of connective tissue at the site of an Arthus reaction is due, not to a direct action of the antigen upon the connective tissue cells, but to interference with the local blood supply. The significant vascular alteration responsible for the focal death of liver cells during protracted anaphylactic reactions may be only an alteration of permeability, which, in itself, could lead to impairment of the nutrition of the liver cells; or there also may occur focal ischaemia, resulting from focal vascular spasm, such as that

observed to occur during focal anaphylactic reactions by Abell and Schenck (10).

We wish to acknowledge the generous coöperation and advice of Dr. G. O. Gey.

## CONCLUSION

Epithelial hepatic cells from Arthus sensitized animals are unaffected by contact with the specific antigen in tissue culture, whether in the presence or absence of specific antibody. In this, they correspond with the other types of cells of the anaphylactically sensitized body that have been tested so far (connective tissue, macrophages, lymphocytes, polymorphonuclear leucocytes, immature marrow cells).

The focal necroses that occur in the liver of the anaphylactic body when the specific antigen circulates in the sinusoidal blood stream are not, therefore, due to a direct action of the antigen upon the epithelial liver cells, as has been postulated, but are probably due to an effect of the antigen upon the vascular endothelium which, in the anaphylactically sensitized body, is well known to be injured by contact with the specific antigen.

## REFERENCES

(1) RICH, A. R., AND LEWIS, MARGARET R.: The Nature of Allergy in Tuberculosis as Revealed by Tissue Culture Studies. Bull. Johns Hopkins Hospital, 1932, **50,** 115.

(2) ARONSON, J. D.: Tissue Culture Studies on the Relation of Tuberculin Reaction to Anaphylaxis and the Arthus Phenomenon. J. Immunol., 1933, **25,** 1.

(3) ARONSON, J. D.: The Specific Cytotoxic Action of Tuberculin in Tissue Culture. J. Experimental Medicine, 1931, **54,** 387.

(4) RICH, A. R., AND FOLLIS, R. H., JR.: Studies on the Site of Sensitivity in the Arthur Phenomenon. Bull. Johns Hopkins Hospital, 1940, **66,** 106–122.

(5) LONGCOPE, W. T.: Cirrhosis of the Liver Produced by Chronic Protein Intoxication. Trans. Ass. Amer. Phys., 1913, **28,** 497–512.

(6) APITZ, K.: Ueber Anaphylactic Organveraenderung bei Kaninchen. Virch. Arch., 1933, **289,** 46–75.

(7) HARTLEY, G., JR., AND LUSHBAUGH, C. C.: Experiment Allergie Focal Necrosis of the Liver. Am. J. Path., 1942, **18,** 323.

(8) GEY, G. O. AND GEY, M. K.: The Maintenance of Human Normal Cells and Tumor Cells in Continuous Culture. Am. J. of Cancer, 1936, **27,** 45–75.

(9) VANDERGRIFT, W. B.: A Dehydrating Fixative for General Use, Including a Description of Techniques and Stains for Paraffin and Celloidin Sections. Bull. of J. H. H., 1942, **71,** 96–111.

(10) MOEN, J. K.: Tissue Culture Studies on Bacterial Hypersensitivity. III. The Persistence in vitro of the Inherent Sensitivity to Tuberculin of Cells from Tuberculous Animals. J. Exper. Med., 1936, **64,** 963.

(11) ABELL, R. G. AND SHENCK, H. P.: Microscopic Observations on the Behavior of Living Blood Vessels of the Rabbit during the Action of Anaphylaxis. Jour. Immunology, 1938, **34,** 195.

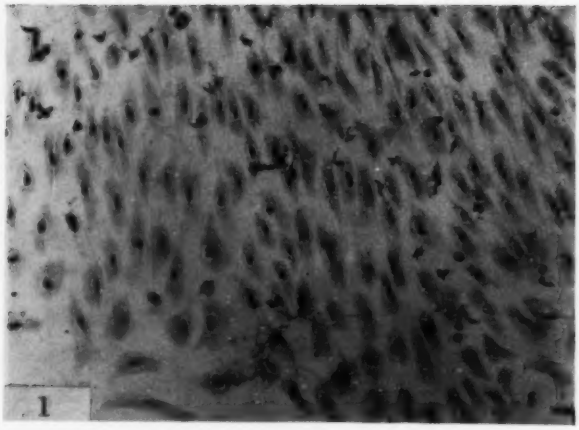
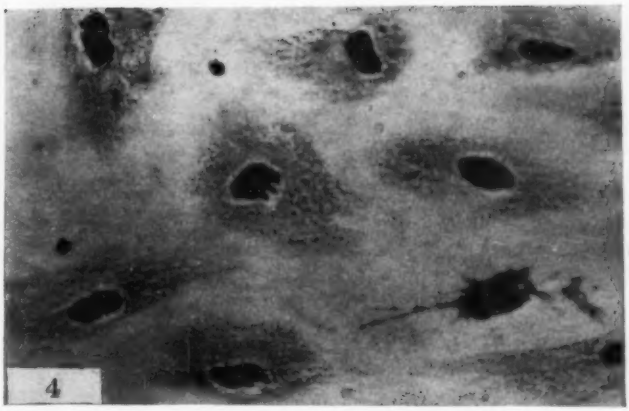
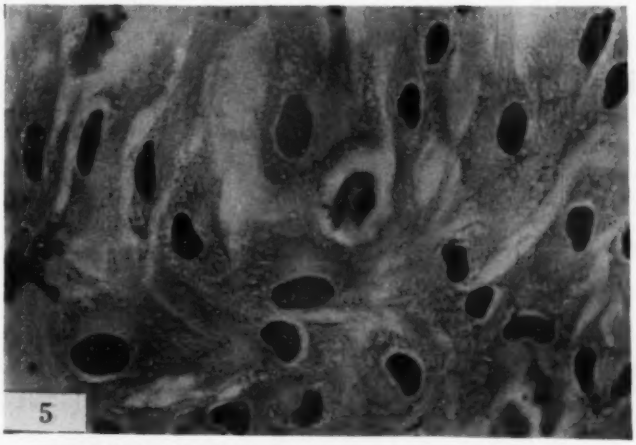


FIG. 1. NORMAL LIVER CELLS IN 30% HORSE SERUM. ×100
FIG. 2. LIVER CELLS FROM A HYPERSENSITIVE ANIMAL IN 30% HORSE SERUM. ×100
FIG. 3. NORMAL GALL BLADDER EPITHELIUM. ×100



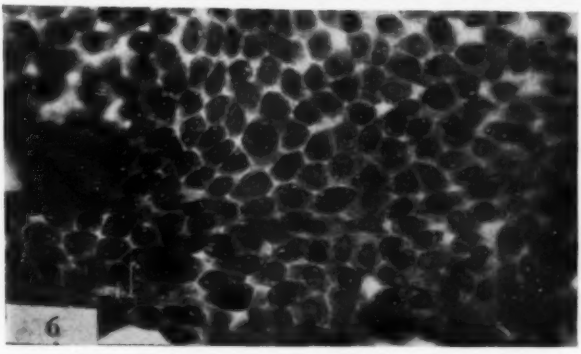




FIG. 4. NORMAL LIVER CELLS IN 30% HORSE SERUM. ×400
FIG. 5. LIVER CELLS FROM A HYPERSENSITIVE ANIMAL IN 30% HORSE SERUM. ×400
FIG. 6. NORMAL GALL BLADDER EPITHELIUM. ×400

# FAILURE OF GLUCOASCORBIC ACID IN THE DIET TO PRODUCE SCURVY IN MICE

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Received for publication July 23, 1948

In 1943, Woolley and Krampitz (1) described in mice and cotton rats a "scurvy-like" condition which was produced by the inclusion of glucoascorbic acid, an analog of ascorbic acid, in the diet. Such animals failed to grow, developed diarrhea and exhibited subcutaneous and gingival hemorrhages. At autopsy "the joints of the legs and ribs were fiery red," and by x-ray examination the bones appeared less dense than those of controls. The addition of ascorbic acid to the diet had no effect on the course of the syndrome, which could be prevented only by the removal of glucoascorbic acid from the ration. Of further interest was the observation that the effects of this material could be produced only on a highly purified diet, not one containing natural food stuffs. Later Woolley (2), using guinea pigs fed a purified diet, found that the addition of ascorbic acid prevented the deleterious manifestations of glucoascorbic acid, and thus concluded that the disease could be "viewed as an ascorbic acid deficiency."

The effects of glucoascorbic acid were further studied by Banerjee and Elvehjem (3) in rats, chicks, and guinea pigs. These investigators confirmed the clinical picture of loss of weight and diarrhea but were unable to find any hemorrhages. The effects of feeding glucoascorbic acid could be prevented by the addition of liver powder to the synthetic diet. Determinations of ascorbic acid concentration in the liver and kidney failed to show any difference with or without the addition of glucoascorbic acid to the diet.

The production of scobutic lesions in an animal such as the mouse or rat would offer a unique chance to study not only changes which are ordinarily not seen in these species but also certain coincident deficiency states, particularly rickets and scurvy which, when they occur together, produce a most confusing morphological picture. It was felt, therefore, that a morphological study of the bones of animals placed on a

glucuoascorbic acid containing diet should be carried out to settle the question as to whether or not scurvy could be produced.

## EXPERIMENTAL

The diet which was employed was identical with the highly purified ration described by Woolley and Krampitz (1). Glucoascorbic acid[1] was added to make a concentration of 5%, which the Rockefeller investigators had found adequate to produce the syndrome. Ten weanling mice were placed on the glucoascorbic acid added ration; five animals served as controls and ate a similar ration without the above supplement.

## RESULTS

The animals on the glucoascorbic acid added ration grew very little; after a few days most of them lost weight. All were dead by the 12th day. They were unkempt; their hair was matted by a sticky brownish material. In contrast the controls grew well. The experimental group developed diarrhea. During life and at autopsy no hemorrhages were seen.

At autopsy no gross changes could be found. Microscopic study of the upper end of the tibia in each animal showed that, beginning at the 3rd day, the cartilage cells appeared to slow their growth activity, the zone of hypertrophic cells becoming narrower. During the remaining week, that is, up until the 12th day, the zone of provisional cartilage cells became narrower and narrower, while osteoblastic activity, as evidenced by bone formation at the cartilage-shaft junction, was greatly retarded. In other words, the picture produced was a nonspecific cessation of both cartilagenous and osteoblastic activity, such as is seen in inanition, and is therefore quite unlike the specific changes which are so pathognomic of skeletal scurvy (4). There were no microscopic hemorrhages in the bone or beneath the periosteum.

## DISCUSSION

The disease scurvy, which occurs as a result of the deprivation of ascorbic acid in certain species, is characterized by a failure of connec-

[1] Furnished through the kindness of Dr. Philip B. Gray of the Wallerstein Laboratories, New York 66, New York.

tive tissue cells, osteoblasts and odontoblasts to deposit their respective intercellular substances: collagen, osteoid and dentine (5). When one finds a disturbance in the formation of one or more of these materials, the diagnosis of scurvy is valid. Thus it is apparent that the most precise way to recognize the disease is to study the tissues of the suspected subject. From the observations reported above, it is apparent that glucoascorbic acid does not produce changes in the bones characteristic of the scorbutic state. The cause for the clinical picture is not clear. The diarrhea and failure to grow are reminiscent of changes produced when excessive quantities of lactose or galactose are added to the diet (6).

SUMMARY

Morphological studies of the bones of young mice which were fed a diet containing 5% glucoascorbic acid failed to reveal any changes characteristic of scurvy; only the effects of acute inanition could be demonstrated.

BIBLIOGRAPHY

1. Woolley, D. W., and Krampitz, L. O.: J. Exp. Med., **78:** 333, 1943.
2. Woolley, D. W.: Fed. Proc., **3:** 97, 1944.
3. Banerjee, S. and Elvehjem, C. A.: Proc. Soc. Exp. Biol. and Med., **60:** 4, 1945.
4. Follis, R. H., Jr.: The Pathology of Nutritional Disease. Charles C. Thomas, Springfield, 1948.
5. Bessey, O. A.:, and Wolbach, S. B.: Physiol. Rev., **22:** 233, 1942.
6. Handler, P.: J. Nutr., **33:** 221, 1947.

# RENAL FAILURE IN EMBOLIC GLOMERULONEPHRITIS AS A COMPLICATION OF SUBACUTE BACTERIAL ENDOCARDITIS

SAUL BOYARSKY, JACK M. BURNETT AND W. HALSEY BARKER[1]

Received for publication July 26, 1948

The recognition of subacute bacterial endocarditis may be difficult in its atypical forms. A combination of septic fever, a cardiac lesion, splenomegaly, anemia, and embolic phenomena is practically diagnostic in the typical case. In the case to be reported the clinical picture of subacute bacterial endocarditis was dominated by the manifestations of renal failure which in turn may have been responsible for the absence of the usual septic temperature curve. Postmortem examination of the kidneys revealed the essential renal lesion to be that of embolic glomerulonephritis, a condition rarely associated by itself with impairment of renal function.

J. H., a 60 year old laborer was admitted to the Surgical Service of the Johns Hopkins Hospital on March 12, 1947, complaining of progressive weight loss of six months duration. He had been referred to the attending surgeon with the diagnosis of gastric carcinoma.

The family history revealed that both parents had died of tuberculosis at the ages of 64 and 74. Two sisters had died of tuberculosis at unstated ages.

The past history was non-contributory. There was no history of chorea, rheumatic fever, or other serious illness.

The present illness had started insidiously six to eight months before admission with weight loss. This had been accompanied by slight dyspnea on exertion and ankle edema. The patient had suffered no abdominal distress, pain, nausea, vomiting or dysphagia at any time.

Several months before admission, the patient had noted the development of nocturia, characterized by the passage of small amounts of normal appearing urine. He had no diurnal frequency, but had been told by his doctor that his kidneys were bad. His vision had started to fail at that time and had become increasingly poor by the time of admission. He had lost 14.5 kilograms. The systemic review was otherwise negative.

*Physical examination:* The temperature was 97°; pulse rate, 78/min.; and respiratory rate, 18/min. Blood pressure readings on three separate occasions were 90/70, 130/90, and 110/80. He weighed 38.6 kilograms and was 170 centimeters tall.

[1] From the Private Ward Medical Service and Department of Pathology, The Johns Hopkins Hospital, Baltimore 5, Maryland.

The patient appeared emaciated and dehydrated. He was resistant and combative. The skin showed innumerable petechiae over the back, extremities and abdomen. It was loose, flabby, and showed marked loss of subcutaneous tissue. The muscles were atrophic and atonic but not tender. Movement of the spine was considerably restricted. There was no generalized or local lymphadenopathy.

The head showed no bony lesions or tenderness. The eyes were sunken and staring with normal pupillary reactions and normal extraocular movements. Ophthalmoscopic examination showed haziness of the media bilaterally. One observer was able to make out several large scattered haemorrhages in both retinae and a large white area surrounded by pigment deposits in the lower temporal quadrant of the right eye.

Examination of the neck showed no goiter or tracheal deviation. The chest was markedly emphysematous. The lungs were clear to physical examination. The heart was slightly enlarged to the left by percussion. The rhythm was regular and slow. The apex impulse was diffuse and slightly displaced to the left. No thrill was felt. A loud, coarse systolic murmur was audible at the apex, but no diastolic murmur could be detected. The peripheral vessels were hard and tortuous. Pulsations in the left foot were diminished.

Abdominal examination showed evidence of marked emaciation, but no masses, rigidity or tenderness. The liver and spleen were not enlarged. The genitalia, prostate, and rectum were essentially normal.

There was no clubbing or cyanosis of the fingers or toes. Aside from the mental status neurological examination was normal.

*Laboratory examinations:* The findings on admission were as follows: Hemoglobin, 8.8 gms. %. Leukocyte count, 15,250. Serological test for syphilis, negative. Urinalysis: specific gravity, 1.010; albumin, +; microscopic, many erythrocytes; 3–4 leukocytes per high power field; no casts. Blood chemistry: non-protein nitrogen, 143 mgm. % and 154 mgm. %; fasting blood sugar, 124 mgm. % and 108 mgm. %; chlorides, 106.2 Meq/L.; $CO_2$ combining power, 14.9 Meq/L.; Bilirubin, less than 0.8 mgm. %.

A chest x-ray showed no evidence of metastases. A gastrointestinal series was reported to be negative. The stomach was described as large, ptosed, but within normal limits.

*Urological consultation:* The patient was examined by a urological consultant whose clinical impression was: marked chronic bilateral pyelonephritis and cicatricial contracture of the vesical orifice with obstructive changes in the bladder.

The intravenous pyelogram showed poor visualization and filling of the upper urinary tract. Cystoscopy showed contraction of the vesicle neck, hypertrophy of the trigone, and trabeculation of the bladder. Retrograde pyelography was done on the right side only and showed flattening and expansion of the minor calices compatible with the shortening and flattening of the pyramids seen in chronic pyelonephritis. There was no evidence of hydronephrosis.

*Hospital course:* The patient was continuously afebrile, but showed no signs of improvement. He remained mentally dull, answered questions slowly, and was

usually uncooperative. A phenolsulfonphthalein test could not be performed because the patient voided on the floor. Repeat urine specimens were difficult to obtain. On the fifth hospital day the NPN had risen to 173 mgm. %. Repeat chest x-rays three days later showed no evidence of significant lesions in the lungs or abnormality of the heart's shadow.

The patient was transferred to the Medical Service with a presumptive diagnosis of chronic pyelonephritis with uremia.

The blood chemistry showed the following changes on the ninth day after admission to the hospital: Non-protein nitrogen, 202 mgm. % and 250 mgm. %. Uric acid, 10.7 mgm. %. Calcium, 9.1 mgm. %. Phosphorus 8.9 mg. %. $CO_2$ combining power, 12.4 Meq/L. Chlorides, 109.2 Meq/L.

The situation was considered hopeless unless a cause for the renal failure amenable to therapy could be discovered. Since chronic pyelonephritis did not satisfactorily explain all the clinical manifestations, diagnostic study was continued. Because of the anemia, purpura, and uremia in the absence of hypertension, multiple myeloma was considered. Further laboratory work was carried out with this possibility in view. The findings of the tenth hospital day were: Erythrocyte count (millions), 2.38. Hemoglobin, 6.7 gms. %. Volume packed RBC, 20 %. Mean corpuscular volume, 84 cu. microns ($N = 87 \pm 5$). Mean corpuscular hemoglobin, 28 microgamma ($N = 29 \pm 2$). Mean corpuscular hemoglobin concentration 33% ($N = 34 \pm 2$). Icterus index, 2. Sedimentation rate (Wintrobe), 43 mm./C.S.R. = 0. Leukocyte count, 10,450. Juvenile neutrophiles, 11%. Polymorphonuclear leukocytes, 83%. Lymphocytes, 2%. Monocytes, 4%. Platelet count, 152,000. Bleeding time, 30 sec. ($N = 60 - 120$). Clotting time, 8 min. (normal). Clot retraction begins, 1 hour (normal). Clot retraction complete, 17 hours (normal). Tourniquet test (Rumpel-Leeds), negative. Blood Smear showed slight hypochromia, poikilocytosis, and rare polychromatophilia.

Sternal puncture was unsatisfactory in that chiefly peripheral blood was obtained. No plasma or myeloma cells were seen in the material examined. Total serum proteins were 5.6 Gms. %, with 2.8 Gms. % albumin and 2.8 Gms. % globulin, giving an A/G ratio of 1.0. X-ray of the skull, ribs and shoulder girdles showed no evidences of bony lesions. Urinalysis showed the following: Bence-Jones protein, negative. Albumin, ++. Specific gravity, 1.011. Microscopic, many leukocytes and erythrocytes.

Since subacute bacterial endocarditis was considered a remote possibility despite the lack of fever, a blood culture was taken on the 9th hospital day. The heart murmur was explained clinically as due to calcification of the mitral valve with organic insufficiency. An electrocardiogram on March 20th showed only low voltage in the limb leads.

On the tenth day after admission the patient suddenly developed clinical signs of a cerebro-vascular accident. His neck was stiff and he soon lapsed into coma. In view of his moribund condition and poor prognosis, the patient was not disturbed for lumbar puncture. He died the following day.

On the day that he died, the blood culture was reported to show a growth of "Alpha Streptococcus."

*Autopsy:* The autopsy (#20493) was performed approximately three hours after death. Examination of the body revealed an extremely emaciated white man weighing only 36.5 kilograms. The entire body was covered by a petechial rash that involved even the conjunctivae and nail beds. Findings of special importance were:

*Heart:* The heart was not large and weighed only 270 grams. The tricuspid, pulmonic, and aortic valves were normal. A large, V-shaped defect in the posterior leaflet of the mitral valve extended from its free edge almost to the valve insertion and measured approximately 15 mm. in diameter. The edge of this defect was covered by smooth endocardium and had chordae tendinae attached to it. There were no fresh or healed vegetations on the valve around this defect. There were soft, brown, friable vegetations on the entire anterior leaflet of the mitral valve and on the left half of the posterior leaflet. The largest of the vegetations was located on the anterior leaflet and measured approximately 15 mm. in diameter. The endocardium of the left auricle above the anterior leaflet of the mitral valve was roughened irregularly, involving an area approximately 20 mm. in diameter. There was a nodular vegetation 5 mm. in diameter in the central portion of this roughened area.

*Urinary system:* The right kidney weighed 110 gm. and the left, 130 gm. The subcapsular surfaces were smooth and several petechiae were noted on these surfaces. On section neither the boundaries of the cortex nor its striations were clearly defined. Several submucosal petechiae were present in the renal pelves and urinary bladder. The calices, renal pelves, and ureters were not dilated. The ureters, urinary bladder, prostate, and urethra were normal with the exception of the submucosal hemorrhages in the urinary bladder.

*Spleen:* The spleen weighed 180 gm. It contained three yellow infarcts, two of them measuring about 6 mm. in diameter and the third, 18 mm. Each was surrounded by a red halo about 2–3 mm. in width.

*Brain:* The lepto-meninges were delicate and pale except over the upper surface of the brain where there were yellowish-brown foci. The blood vessels at the base of the brain were delicate and free of arteriosclerotic changes. There was no evidence of a "pressure cone."

On the cut surface of most of the sections through the brain there were petechial hemorrhages in both the white and gray matter. There were several small but distinct areas of softening in the anterior portion of the left corpus striatum. Deep in the left anterior sulcus there was a vessel that appeared to contain a relatively fresh thrombus. In the cortex beneath this thrombosed vessel there was a round hemorrhagic area, approximately 8 mm. in diameter.

*Aorta:* The aorta was elastic and had a smooth, ivory-like intima throughout. There was a tiny opening less than 1 mm. in diameter in the ductus arteriosus.

*Microscopic studies: Heart:* Section through the infected portion of the mitral valve and the left auricle revealed organizing vegetations covered by fibrin.

Clumps of Gram-positive cocci were visible near the surface of the vegetations. Throughout the myocardium, pancreas, spleen, and brain there were myriads of microscopic infarcts. There were a number of mycotic aneurysms in the small blood vessels of the heart, pancreas, spleen, and brain. No fresh or old Aschoff bodies were seen in the myocardium or in the left auricular endocardium. No evidence of inflammatory changes were seen in a section through the edge of the mitral valve defect.

*Kidneys:* Sections through the kidneys showed extensive and typical lesions of embolic glomerulonephritis. Although many of the glomeruli appeared normal, the remainder showed embolic phenomena varying from hyaline and fibrous changes in one or more capillary loops to complete fibrosis of a glomerulus. Other glomeruli contained fibrous and epithelial crescents; erythrocytes were seen within the capsules of others. These distinctly focal lesions contrast with the rather uniform glomerular lesions seen in the diffuse subacute glomerular nephritis associated with Alpha streptococcus endocarditis. Many of the tubules contained granular casts, and there were foci of round cells in the renal parenchyma. No organisms were seen in bacterial stains of the kidneys. Bacteria were stained in the large infarcts of the spleen and in the large thrombosed meningeal vessel described grossly in the left anterior sulcus.

*Postmortem bacteriological studies:* From a culture of the vegetation on the mitral valve (which was contaminated in removal) Alpha Streptococcus mitis was isolated, along with several common intestinal contaminants. A direct smear of the vegetation itself revealed numerous Gram-positive cocci in pairs and chains. From the heart's blood, a pure culture of Group B Beta hemolytic streptococcus opportunis was isolated, which probably represented a terminal invader.

*The complete final anatomical diagnosis was:* Congenital malformation of the mitral valve. Patent ductus arteriosus. Bacterial endocarditis (Alpha Streptococcus mitis) with vegetations on the mitral valve and wall of the left ventricle. Emboli and minute infarcts in the myocardium, spleen, pancreas, and brain; mycotic aneurysms in the coronary, pancreatic, and cerebral arteries; petechial hemorrhages in the skin, fingernail beds, renal pelves, submucosa of the urinary bladder, conjunctivae, and retina. Extensive embolic glomerulonephritis. Septic choroiditis. Hyperplasia of bone marrow. Purulent bronchitis and lobular penumonia. Emaciation. Partial optic atrophy and scars of old choroiditis. Atrophy of testes. Fibrous adhesions over lower lobe of right lung. Calcified nodules in the lungs, hilar lymph nodes, and liver. Polyps of the colon.

There was clearly a congenital malformation of the mitral valve, in view of the fact that the deformed valve was normal histologically, and chordae tendinae were attached to the free edge of the congenital defect. Since there were no rheumatic lesions in the heart, the bacterial endocarditis was evidently associated with a congenitally malformed mitral valve. The susceptibility of malformed valves to endocarditis is well known. Embolic nephritis sufficiently extensive to produce renal failure is rare in subacute bacterial endocarditis. As evidenced by the purpuric cutaneous petechiae and myriads of microscopic infarcts in the myocardium,

pancreas, and brain, there were obviously widespread minute emboli throughout the entire body.

## DISCUSSION

Fever is generally considered to be a cardinal sign of subacute bacterial endocarditis but, as several authors have stated (1, 2, 3, 4, 5), it may be absent especially when renal insufficiency develops. An interesting feature of the case just reported was the lack of febrile response during the period of observation.

The lesions of focal embolic nephritis were first described by Lohlein in 1910 (6). Baehr and his group of workers, between 1912 and 1923, published a classic series of papers (7, 8, 9, 10, 11, 12, 13) contributing to our knowledge of this condition. Further studies were made by Gaskell (14) in 1913 and in 1932 by Bell (15). As frequently as this lesion accompanies subacute bacterial endocarditis, it is rarely extensive enough to cause renal failure. Primary renal failure developing during the course of bacterial endocarditis is usually the result of diffuse glomerulonephritis (11, 16, 17). Libman is quoted by Fishberg (17) as saying that in 800 cases of subacute bacterial endocarditis observed he had never seen a case of uremia due to embolic glomerulonephritis.

A review of the records of the Johns Hopkins Hospital from 1925 through 1945 reveals 83 autopsied cases in which there was either a diagnosis of Alpha streptococcus endocarditis or of subacute bacterial endocarditis. In only nine of these autopsied cases was there a terminal rise above 80 mgm. % in the blood non-protein nitrogen. Postmortem examination of the kidneys from these nine cases gave the following picture: Chronic diffuse glomerulonephritis, one case. Acute diffuse glomerulonephritis, one case. Subacute diffuse glomerulonephritis, three cases. Essentially normal kidneys, two cases. Focal embolic nephritis, two cases. The two patients with focal embolic glomerulonephritis did not have the extensive renal lesions noted in the present case, and both of them, together with the two patients having apparently normal kidneys, were in frank heart failure terminally, thus accounting for their final azotemia. Therefore, in none of these nine cases could focal embolic glomerulonephritis be considered a major factor in their terminal azotemia.

That hypertension may be absent in cases of "renal failure" in subacute bacterial endocarditis has been mentioned by Libman (18), although he did not specify the type of renal failure or the type of renal lesion, and he gave no supporting figures for his statement.

The absence of hypertension in cases of extensive subacute glomerulonephritis associated with subacute bacterial endocarditis is a matter of interest, since hypertension is, of course, the rule in subacute glomerulonephritis in the absence of subacute bacterial endocarditis. It is conceivable that the absence of hypertension in such cases might be due to weakening of the myocardium as a result of widespread emboli from the vegetation. In four of the five cases of diffuse glomerulonephritis listed above, hypertension was not observed at any time during the hospital stay of the patient. On the other hand, the one case of chronic diffuse glomerulonephritis did have hypertension when admitted to the ward. Death occurred nine days later with a sustained hypertension and clinical signs of uremia (Nonprotein nitrogen before death = 215 mg.%).

The three above-mentioned cases with subacute diffuse glomerulonephritis, in which the blood pressure was not elevated, did have many small myocardial infarctions from such embolization, while the case of chronic diffuse glomerulonephritis in which the blood pressure remained elevated had a normal appearing myocardium.

Bell (15) has reported five cases of "uremia" (details not given) due to multiple glomerular embolization in a series of 108 autopsied cases of subacute bacterial endocarditis. Other cases of "uremia" due to embolic glomerulonephritis have been mentioned by Middleton and Burke (19) and Sautereau (20). Hamman and Brown mention a case seen by Bloomfield (21).

At least 16 cases of "uremia" caused by focal embolic glomerulonephritis have been reported previous to the present case.

## SUMMARY

A case of subacute bacterial endocarditis implanted upon a congenitally deformed mitral valve, with multiple embolic phenomena and uremia due to focal embolic glomerulonephritis, is presented. In this case unusual features were (1) the absence of fever during the period of observation, (2) the complete absence of hypertension in the

face of marked renal failure, and (3) severe uremia caused by focal embolic glomerulonephritis.

## BIBLIOGRAPHY


1. Horder, T. J.: Infectious Endocarditis. Quart, J. Med. Oxford, **2:** 289, 1908–1909.
2. Le Noir, P., and Baize, P.: Le Rein dans L'Endocardite Maligne Lente Formes rénales de la Maladie d'Osler. Presse Med., **36:** 1234, 1928.
3. Le Noir, Baize, P., and Boquien: Endocardite Maligne Lente Simulant une Néphrite Hématurique. Evolution Apyrétique, Bull. et Mém. Soc. Med. des Hosp. de Paris, **52:** 1041, 1928.
4. Libman, E.: Clinical Features of Subacute Streptococcus (and Influenzal) Endocarditis in the Bacterial Stage. Med. Clin. N. Am., **2:** 117, 1918–1919.
5. Caussade, G., and Foucart, A.: Endocardite Aigue. Mort par Néphrite aigue, avec Anurie et Azotémie. Bull. et Mém. Soc. Med. des Hospitaux de Paris, **45:** 581, 1921.
6. Löhlein, M.: Uber Hämorrhagische Nierenaffektionen bei Chronischer Ulzeröser Endokarditis. Med. Klin. Berlin, **6:** 375–379, 1910.
7. Baehr, G.: Glomerular Lesions of Subacute Bacterial Endocarditis. J. Exp. Med., **15:** 330, 1912.
8. Baehr, G.: Glomerular Lesions of Subacute Bacterial Endocarditis. Am. J. Med. Sc., **144:** 327, 1912.
9. Libman, E.: The Clinical Features of Cases of Subacute Bacterial Endocarditis that have Spontaneously Become Bacteria-Free. Am. J. Med. Sc., **146:** 625, 1913.
10. Baehr, G., and Lande, H.: Glomerulonephritis as a Complication of Subacute Streptococcus Endocarditis. J. A. M. A., **75:** 789, 1920.
11. Baehr, G.: The Significance of the Embolic Glomerular Lesions of Subacute Streptococcus Endocarditis. Arch. Int. Med., **27:** 262, 1921.
12. Baehr, G., and Sacks, B. The Occurrence of Glomerulonephritis in Association with Verrucous Endocarditis. Proc. N. Y. Path. Soc., **23:** 64, 1923.
13. Baehr, G.: Renal Complications of Endocarditis. Trans. Assoc. Am. Physicians, **46:** 87, 1931.
14. Gaskell, J. F.: The Lesions of the Kidney in Ulcerative Endocarditis. Proc. Royal. Soc. Med., 1913–1914, vii, Path. Sect. 109.
15. Bell, E. T.: Glomerular Lesions Associated with Endocarditis. Am. J. Path., **8:** 639, 1932.
16. Bell, E. T.: Renal Diseases. Lea and Febiger, Philadelphia, 1946, 158–163.
17. Fishberg, A. M.: Hypertension and Nephritis. Lea and Febiger, Philadelphia, 1939.

18. LIBMAN, E., AND FRIEDBERG, C. K.: Subacute Bacterial Endocarditis. Oxford Med., Oxford Univ. Press N. Y., **2:** 346, 1941.
19. MIDDLETON, W. S., AND BURKE, M.: Strep-Viridans Endocarditis Lenta. Am. J. Med. Sc., **198:** 301, 1939.
20. SAUTEREAU, P.: L'Endocarditi Maligne a Forme D'Insuffisance Aortique. Thèse (Lyon) 1925.
21. HAMMAN, L., AND BROWN, T. McP.: Subacute Bacterial Endocarditis. Internat. Clin., **3:** 33, 1937.

# STUDIES ON THE HEMOLYSIS OF HUMAN ERYTHROCYTES BY HOMOLOGOUS COMPLEMENT IN THE PRESENCE OF TANNIC ACID[1]

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Received for publication July 30, 1948

The existence of so-called artificial amboceptors, which have the property of "sensitizing" red cells for lysis by complement, has long been recognized. Landsteiner (1) showed that erythrocytes were agglutinated by small amounts of silicic acid and became hemolyzed upon the subsequent addition of complement. Reiner and Fischer (2) demonstrated that tannic acid exerted a similar action on red cells. Neufeld and Etinger-Tulcyznska (3) confirmed this observation, and extended the list of "sensitizers" to include alum and the salts of several heavy metals. Freund (4) showed that tannic acid rendered erythrocytes more susceptible to phagocytosis. Gordon and Thompson (5, 6) reported that tannic acid and the salts of certain heavy metals enhanced the phagocytosis of staphylococci and E. typhosus. The mechanism by which these substances produced their effects has never been made clear. It has been assumed that they damage the cell membrane, but the nature of this damage is not understood.

Very little is known about the mechanism by which hemolysis occurs when red cells, specific antibody, and complement are combined. From a theoretical viewpoint, this reaction is of fundamental importance. Aside from its relation to problems of red cell function and destruction, the hemolytic system offers a relatively simple model with which to study the general problem of tissue injury by immune mechanisms. The deficiencies in our understanding of this problem are basic. Much has been learned about the nature and properties

[1] Aided by a grant from the Life Insurance Medical Research Fund.

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of antibody and complement, but little is known about the actual function of these agents in the production of tissue damage.

In attempting to study the rôle of complement in hemolysis, one is confined to a limited range of experimental manipulation by the complexity and variability of the reagents involved; in the system ordinarily employed, the red cells are derived from one species (sheep), complement from another (guinea pig), and the antibody from a third (rabbit). The present investigation of the action of tannic acid as an artificial "sensitizer" represents an attempt to simplify the conditions under which the lytic action of complement can be observed, by substituting a known chemical reagent for antibody, and by using homologous red cells and complement.

In preliminary experiments using physiological saline solution as a diluent for all reagents, hemolysis of red cells from various species could be elicited only occasionally with tannic acid and complement, and, when lysis did occur, it was usually incomplete and required high concentrations of complement. When, however, the salt concentration was decreased, a marked enhancement of the hemolytic action of tannic acid with human red cells and complement was observed, although the cells and complement of other species still yielded irregular results. In view of this finding, the subsequent experiments on which this report is based were carried out for the most part with human red cells and complement using 0.7 percent sodium chloride instead of the customary physiological saline as diluent. It was found that tannic acid, under the conditions to be described, caused human erythrocytes to undergo lysis with complement from the same or other human subjects. It was possible to titrate human complement by this means with reproducible endpoints similar to those obtained in titrations with amboceptor-sensitized sheep cells. Moreover, human red cells and complement could be employed with tannic acid as an indicator system in complement fixation tests. A preliminary report of these observations has been published (7). The present paper is concerned with a detailed description of the conditions which are necessary for hemolysis with tannic acid and complement, and with certain observations which bear upon the mechanism of the phenomenon.

## MATERIALS AND METHODS

*Diluent.* Unless otherwise stated, the diluent for all reagents consisted of 0.7 percent sodium chloride in distilled water.

*Cells and Complement.* Venous blood was obtained from normal human subjects, a portion being added to oxalate crystals and the remainder allowed to clot at room temperature. Cells from the oxalated blood were washed three times in at least 20 volumes of 0.85 percent saline and then taken up in sufficient 0.7 percent saline to make a one percent suspension by volume. The clotted blood was centrifuged at 2000 R.P.M. for 10 minutes, and the serum so obtained kept in an ice bath until used as complement. Fresh lots of cells and complement were prepared each day.

*Tannic Acid.* A single lot of crystalline tannic acid[3] was used in all experiments to be described. A one percent solution in 0.7 percent sodium chloride was prepared each day and various dilutions of this were tested for the optimal concentration in the hemolytic system under study.

*Sensitization of Cells.* Two parts of the red cell suspension were added to one part of tannic acid and the mixture allowed to stand at room temperature for 15 minutes or longer before the addition of complement or other reagents. It was not necessary to adjust the pH of the tannic acid solution before sensitization, since the mixture of cells and tannic acid, in the concentrations usually employed, had a pH of 7.0 or slightly higher.

*Titration of Complement.* Human serum was added to tubes in volumes of 0.07, 0.06, 0.05, 0.04, 0.03, and 0.02 cc. and the volume was made up to 0.2 cc. with 0.7 percent NaCl. 0.6 cc. of sensitized cells was then added and the total volume increased to 1.2 cc. by the addition of 0.4 cc. of 0.7 percent sodium chloride. The tubes were then placed in a water bath at 37°C. for 30 minutes after which readings of the degree of hemolysis were made. The smallest amount of complement yielding complete or four plus hemolysis was taken as the endpoint and represented one unit of complement activity. In studies of the effect of other substances on hemolysis, the additional

[3] C. P. Crystalline Tannic Acid, Lot No. 1, 1945. J. T. Baker Co., Phillipsburg, New Jersey.

reagents were employed in volumes of 0.2 cc. in place of an equivalent volume of the sodium chloride solution.

## EXPERIMENTAL

### *Factors Influencing the Reaction*

*The Effect of Salt Concentration on Hemolysis.* Various concentrations of sodium chloride in distilled water were employed as diluent for all reagents involved, and the endpoints of complement activity

TABLE I

*The Effect of NaCl Concentration on the Hemolysis of Human Cells by Human Complement and 0.06 Percent Tannic Acid*

| NaCl in diluent | Volume of complement (cc.) | | | | | | | Control† |
|---|---|---|---|---|---|---|---|---|
| | 0.06‡ | 0.05 | 0.04 | 0.03 | 0.02 | 0.01 | 0.00 | |
| *percent* | | | | | | | | |
| 0.60 | ++++* | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 |
| 0.65 | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 |
| 0.70 | ++++ | ++++ | ++++ | ++++ | +++ | 0 | 0 | 0 |
| 0.75 | ++++ | ++++ | +++ | ++ | 0 | 0 | 0 | 0 |
| 0.80 | ++++ | ++++ | ++ | 0 | 0 | 0 | 0 | 0 |
| 0.85 | ++ | + | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.90 | + | + | 0 | 0 | 0 | 0 | 0 | 0 |

* In this and following tables, symbols indicate: ++++, complete hemolysis; +, slight hemolysis; 0, no hemolysis.

† Control: 0.06 cc. complement with unsensitized cells.

‡ Figures indicate amount of complement in final volume of 0.2 cc.

determined. In these experiments, tannic acid was used in a concentration of 0.06 percent, and red cells in a one percent suspension. The results of a representative experiment are shown in Table I. It will be seen that the optimal salt concentration for the reaction was in the vicinity of 0.7 percent. Hemolysis was almost completely inhibited by sodium chloride concentrations of 0.85 or 0.9 percent, while concentrations of 0.6 percent were less effective than 0.7 percent. At 0.5 percent or less, hemolysis due to hypotonicity occurred. Because of these findings, the diluent employed for all reagents in the experiments to be recorded consisted of 0.7 percent sodium chloride, unless otherwise stated.

*Effect of Salt Concentration on Sensitization of Red Cells.* It was of interest to determine whether varying concentrations of sodium chloride exerted an effect on the capacity of tannic acid to sensitize the red cells, independent of the effect on hemolysis itself. Accordingly, red cells were suspended in different concentrations of sodium chloride and exposed to 0.06 percent tannic acid dissolved in similar salt concentrations. After the mixtures had stood together for 30 minutes, the cells were separated by centrifugation and resuspended in 0.7 percent sodium chloride. A titration of complement using these cells was then made, with the results shown in Table II. It will be seen that cells which had been exposed to tannic acid in the pres-

TABLE II

*The Effect of NaCl Concentration on the Sensitization of Human Red Cells by 0.06 Percent Tannic Acid*

| NaCl in diluent* | Volume of complement (cc.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.1 | 0.08 | 0.06 | 0.04 | 0.02 | 0.01 | 0.00 |
| *percent* | | | | | | | |
| 0.70 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 0 |
| 1.5 | + | + | ± | ± | ± | ± | 0 |

* Red cells and tannic acid were mixed together in NaCl solution of the concentrations indicated. After 30 minutes, the cells were removed, washed, and tested with complement in 0.7 percent NaCl.

ence of 1.5 percent saline were not lyzed by complement and presumably had not been sensitized, while cells which had been in an environment of 0.7 percent NaCl were hemolyzed.

These results indicated that the sensitization of red cells by tannic acid was prevented in excessive concentrations of sodium chloride. At the same time, it was demonstrated that red cells adequately sensitized with tannic acid could be separated from the tannic acid and still retain their susceptibility to lysis by complement. This property of red cells will be dealt with below, where it will be shown that an actual adsorption of tannic acid by red cells occurs.

*Effect of Glucose as Diluent for the Reagents Involved.* The effect of substituting glucose solutions for NaCl as diluent for the reagents in the test was studied. Red cells which had been washed in physiological saline were resuspended in glucose solutions of 4, 5 and 6

percent and exposed to 0.06 percent tannic acid dissolved in similar concentrations of glucose. Complement diluted in similar solutions was then added. The results are shown in Table III. It will be seen that hemolysis occurred with tannic acid and complement when sodium chloride was omitted from the diluting fluid, although the activity of complement was less than in 0.7 percent saline.

The ability of tannic acid to sensitize cells in a diluent of glucose was studied. Red cells washed three times in physiological saline were suspended to one percent in 5 percent glucose. 0.06 percent tannic acid in the same diluent was added, and the mixtures allowed to stand for 30 minutes. The cells were then washed once in 5 percent glucose and resuspended in 0.7 percent sodium chloride. This

TABLE III

*Effect of Glucose on Hemolysis of Cells by Complement and 0.06 Percent Tannic Acid*

| DILUENT* | VOLUME OF COMPLEMENT (CC.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.08 | 0.06 | 0.04 | 0.02 | 0.00 |
| 0.7% NaCl | ++++ | ++++ | ++++ | ++++ | 0 | 0 |
| 4% Glucose | ++++ | ++++ | ++++ | 0 | 0 | 0 |
| 5% Glucose | ++++ | ++++ | +++ | 0 | 0 | 0 |
| 6% Glucose | +++ | +++ | +++ | 0 | 0 | 0 |

* All reagents prepared in diluent indicated.

suspension was used in a titration of complement. Red cells so treated were as susceptible to lysis by complement as were cells sensitized in 0.7 percent NaCl showing that complete sensitization occurred in a diluent of glucose.

*The Optimal Concentration of Tannic Acid.* Various concentrations of tannic acid, dissolved in 0.7 percent saline, were added to a one percent suspension of red cells, and the mixture allowed to stand for 30 minutes. Titrations of complement were then made with each of these mixtures. The results are shown in Table IV. The range of effective tannic acid was narrow, lying between 0.12 and 0.015 percent. Above or below these concentrations, hemolysis failed to occur. For the experiments to be reported, a dilution of 0.06 percent tannic acid was used unless otherwise stated.

*The Optimal Concentration of Red Cell Suspensions.* Various con-

centrations of red cells in 0.7 percent saline were compared for their susceptibility to lysis in the presence of tannic acid and complement. The optimal concentration of cells was found to be one percent. Lower concentrations of cells rendered the reading of endpoints of hemolysis unreliable. The use of higher concentrations resulted in

TABLE IV

*Effect of Concentration of Tannic Acid on Hemolysis of Cells by Complement*

| CONCENTRATION OF TANNIC ACID | VOLUME OF COMPLEMENT (CC.) | | | | |
|---|---|---|---|---|---|
| | 0.1 | 0.08 | 0.06 | 0.04 | 0.02 |
| *percent* | | | | | |
| 0.1 | ++++ | ++++ | ++++ | + | 0 |
| 0.06 | ++++ | ++++ | ++++ | ++++ | 0 |
| 0.03 | ++++ | ++++ | ++++ | ++++ | 0 |
| 0.015 | +++ | +++ | +++ | +++∓ | ++ |
| 0.008 | 0 | 0 | 0 | 0 | 0 |

TABLE V

*Effect of pH on Sensitization of Red Cells by 0.06 Percent Tannic Acid*

| pH* | VOLUME OF COMPLEMENT (CC.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.07 | 0.05 | 0.03 | 0.01 | 0.008 | 0.006 | 0.004 | 0.00 |
| 6.0 | ++ | ++ | +++ | ++ | ++ | ++ | ++ | ++ |
| 6.5 | +++ | +++ | ++++ | +++ | + | + | 0 | 0 |
| 7.0 | ++++ | ++++ | ++++ | ++++ | ++++ | + | + | 0 |
| 7.5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 |
| 8.0 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | + | 0 |
| 8.5 | ++++ | ++++ | ++++ | ++++ | ++++ | + | 0 | 0 |

* Figures refer to pH in mixtures of cells and tannic acid. Cells were separated after 30 minutes and tested with complement.

hemolysis which was usually incomplete, regardless of the amounts of tannic acid or complement.

*The Effect of pH on the Sensitization of Cells.* Samples of a one percent suspension of red cells were adjusted to various pH values by the addition of 0.185 M hydrochloric acid or 0.185 M sodium hydroxide. Tannic acid solutions, adjusted to similar pH values, were added to the cells and the mixtures allowed to stand for 30 minutes. The cells were then separated by centrifugation and resuspended to the

appropriate volume in 0.7 percent saline. The susceptibility of these cell suspensions to lysis by complement was then tested with the results shown in Table V. It will be noted that the optimal pH for sensitization of red cells by tannic acid was between 7.0 and 8.0.

*The Effect of Time of Exposure on the Sensitization of Red Cells by Tannic Acid.* To samples of red cell suspension were added 0.06 and 0.015 percent tannic acid respectively, and the mixtures allowed to stand for varying periods of time before being tested for hemolysis by the addition of complement. The results are shown in Table VI.

TABLE VI

*Time Required for Sensitization of Red Cells by Tannic Acid*

| CONCENTRATION OF TANNIC ACID | PERIOD OF SENSITIZATION | VOLUME OF COMPLEMENT (CC.) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.1 | 0.06 | 0.05 | 0.04 | 0.03 | 0.02 |
| *percent* | | | | | | | |
| 0.06 | 1 minute | | ++++ | +++∓ | + | 0 | 0 |
| | 5 minutes | | ++++ | ++++ | ++++ | ++++ | + |
| | 15 minutes | | ++++ | ++++ | ++++ | ++++ | 0 |
| | 60 minutes | | ++++ | ++++ | ++++ | ++++ | 0 |
| | 24 hours | | ++++ | ++++ | ++++ | ++++ | ++ |
| 0.015 | 1 minute | ++ | ++ | ++ | + | + | ± |
| | 15 minutes | ++ | ++++ | +++ | ++ | + | + |
| | 30 minutes | +++∓ | +++∓ | +++ | ++ | + | + |
| | 60 minutes | ++++ | ++++ | ++++ | ++++ | +++ | ++ |

With 0.06 percent tannic acid, hemolysis by the smallest effective amounts of complement was demonstrable at the end of 5 minutes, indicating that sensitization of the red cells was completed within this period. With a lesser concentration of tannic acid (0.015 percent), however, sensitization was not complete until the cells had been exposed to tannic acid for at least 30 minutes. In other experiments, similar observations were made, i.e., within the effective range of tannic acid, the rate of sensitization was slower with the lower concentrations of tannic acid. Once sensitization of red cells had occurred, the cell suspensions retained their susceptibility to lysis by complement for a period of 24 hours without change.

*The Effect of Using Complement and Cells of Different Individuals*

*and Different Species.* The degree of hemolysis produced by the complement of one human subject against suspensions of sensitized cells from other individuals was tested. In cross tests between four individuals, no significant difference was observed between the hemolytic capacity of a sample of human complement for the cells of the same or other persons, or between the susceptibility to lysis of different lots of sensitized human cells.

The results of testing complement and tannic-acid treated cells obtained from other species were quite different. Human serum could not be tested satisfactorily against the red cells of rabbits or guinea pigs because of the frequent occurrence of natural lysins for these cells in human serum. Rabbit serum was completely lacking in hemolytic property for homologous cells treated with tannic acid, and produced only slight lysis of human and guinea pig cells. Guinea pig serum contained a natural lysin for human cells, and exhibited only slight hemolysis with tannic-acid treated guinea pig cells. Human, rabbit, and guinea pig serum were without significant hemolytic effect on sheep or mouse cells treated with tannic acid.

These results indicated that under the conditions employed in the test, satisfactory and reproducible hemolysis could be obtained only in the system consisting of human red cells and human complement. It is possible that the failure to demonstrate hemolysis with complement and cells from other species may be due to a greater susceptibility of the complement in these species to a primary anticomplementary action of tannic acid. Experiments which support this explanation will be described below.

*The Effect of Variation in the Order of Mixing the Reagents.* In order to elicit hemolysis, it was necessary that red cells and tannic acid be allowed to stand together for a sufficient time to permit the sensitization of cells to take place. When complement was added to the cell suspension before tannic acid, hemolysis did not take place. When complement, cells, and tannic acid were mixed simultaneously, hemolysis likewise failed to occur. If small amounts of serum, or other protein solutions, were exposed to tannic acid prior to the addition of cells, sensitization of the cells was prevented. Moreover, if cells were used which had been inadequately washed, sensitization by tannic acid could not be produced. Studies on the mechanism by which serum inhibits sensitization will be described below.

### *Evidence for Complement as the Agent Causing Hemolysis*

In order to be certain that the hemolysis of tannic acid-sensitized cells by serum was due to complement, and not to other lytic substances in the serum, a study of the properties of the serum factor was undertaken. The following pertinent observations were made. 1) Heating fresh serum at 56°C. for 30 minutes caused complete inactivation of its lytic action. Inactivation at this temperature is a characteristic feature of complement. 2) The addition of heparin or congo red to serum prevented the lysis of sensitized cells. These substances are known to be anticomplementary. 3) Specific complement fixation by three antigen-antibody combinations was demonstrated, using human complement and red cells sensitized with tannic acid as the lytic system. A detailed account of this experiment is given elsewhere (7). 4) The titer of complement in several samples of human serum was determined in simultaneous tests with a) human cells sensitized with tannic acid, and b) sheep cells sensitized with rabbit amboceptor. As is shown in Table VII, the endpoints of complement activity as determined by the two methods were indistinguishable.

TABLE VII

*Titration of Human Complement with Tannic Acid-Sensitized Human Cells and Amboceptor-Sensitized Sheep Cells*

| CELLS | VOLUME OF COMPLEMENT (CC.) | | | | |
|---|---|---|---|---|---|
| | 0.08 | 0.06 | 0.04 | 0.02 | 0.01 |
| Human cells and tannic acid* | ++++ | ++++ | ++++ | ++ | 0 |
| Sheep cells and amboceptor† | ++++ | ++++ | ++++ | ++ | 0 |

* 0.06% tannic acid.

† 4 units of rabbit amboceptor.

The foregoing observations are regarded as sufficiently strong evidence to permit the assumption that the lysis of tannic acid-sensitized cells is due to the complement.

### *The Adsorption of Tannic Acid by Red Cells*

In order to determine whether the sensitization of red cells for hemolysis by complement involved the adsorption of tannic acid by the cells, the following experiment was performed.

Two volumes of a suspension of red cells were mixed with one volume of tannic acid solution. Immediately after mixing, and at intervals of 15, 30, and 60 minutes thereafter, aliquot portions of the suspension were centrifuged at 1000 R.P.M. for 5 minutes. The sedimented cells were taken up in NaCl solution, washed once by centrifugation, and resuspended to the original volume of the mixture. These suspensions were then tested for their susceptibility to the lytic action of complement by mixing 0.6 cc. of each suspension with 0.2 cc. of complement dilutions and 0.4 cc. of NaCl solution. At the same time, the supernatant fluid obtained after centrifugation of each mixture

TABLE VIII

*The Susceptibility of Human Red Cells to Lysis by Complement, When the Cells were Mixed with 0.06 Percent Tannic Acid Solution and Then Removed from the Solution by Centrifugation*

| PERIOD OF EXPOSURE* | VOLUME OF COMPLEMENT (CC.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.08 | 0.06 | 0.04 | 0.02 | 0.01 |
| *minutes* | | | | | | |
| 1 | +++ | +++ | ++ | 0 | 0 | 0 |
| 15 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| 30 | ++++ | ++++ | ++++ | ++++ | ++++ | ++∓ |
| 60 | ++++ | ++++ | ++++ | ++++ | ++++ | ++∓ |

* The indicated periods refer to the time of exposure of red cells to 0.06 percent tannic acid, before cells were removed by centrifugation and tested with complement.

of cells and tannic acid was tested for its capacity to sensitize untreated cells. Chemical estimations were made of the amount of tannic acid in the supernatant fluids as compared with the amount in the original solution.

As is shown in Table VIII, red cells retained their susceptibility to lysis by complement after they had been removed from the tannic acid solution and washed. The susceptibility of cells which were removed immediately after exposure to tannic acid was considerably less than that of cells which had remained in tannic acid 15 minutes or longer. This finding was in agreement with the previous observation that an optimal time of 5 to 15 minutes is required for sensitization of red cells.

The amount of tannic acid remaining in the supernatant fluid after

the removal of red cells was less than that in the original solution, and decreased the longer the cells and tannic acid were allowed to stand together. This finding is illustrated in Table IX, in which it will be seen that the capacity of the supernatant fluid to sensitize normal cells was less than that of the original tannic acid solution in each instance, with the supernatant fluid of the mixture which had been kept for 60 minutes before centrifugation showing the least ability to sensitize. Moreover, the amount of free tannic acid as determined colorimetrically[4] was also decreased by exposure to red cells. The supernate from the mixture kept for 30 minutes contained

TABLE IX

*The Diminution in the Sensitizing Action of 0.06 Percent Tannic Acid Solution, When Human Red Cells Were Mixed with the Solution and Then Removed by Centrifugation*

| PERIOD OF EXPOSURE* | VOLUME OF COMPLEMENT (CC.) | | | | |
|---|---|---|---|---|---|
| | 0.1 | 0.08 | 0.06 | 0.04 | 0.02 |
| *minutes* | | | | | |
| 1 | ++++ | ++++ | ++++ | ++++ | 0 |
| 15 | +++ | +++ | ++ | ++ | 0 |
| 30 | ++ | ++ | + | + | 0 |
| 60 | ++ | + | + | 0 | 0 |

* The indicated periods refer to the time of exposure of red cells to 0.06 percent tannic acid, before cells were removed and the supernatant fluid tested with new cells and complement.

29 mg. per cc. as contrasted with 40 mg. per cc. for the original solution of tannic acid.

The foregoing observations indicated that the sensitization of red cells by tannic acid was associated with the adsorption of this substance by the cells.

### *The Fixation of Tannic Acid by Serum*

It was found that the order in which red cells, tannic acid, and complement were combined was an important factor in the production of hemolysis. When tannic acid and red cells were mixed and allowed

[4] Colorimetric determinations of tannic acid were performed after the method described by Greissmayer (8).

to stand for 15 minutes or longer before addition of complement, hemolysis invariably occurred. However, when tannic acid and complement were mixed first and then added to the cells, or when all three were placed together simultaneously, hemolysis did not take place.

Two possible explanations for this observation were considered: 1) that tannic acid, before its adsorption by the cells, produced an irreversible anticomplementary effect on the serum, thus preventing the lytic action of the latter once the cells had become sensitized; or 2) that in the presence of complement, tannic acid was kept from the cells by virtue of an affinity between tannic acid and serum. Choice between these alternative explanations was based on the following experiments.

The anticomplementary effect of tannic acid was tested with human and guinea pig complement, using sheep cells sensitized with rabbit amboceptor as the hemolytic system. Physiological saline was used as diluent for all reagents. Decreasing amounts of complement in a volume of 0.2 cc. were mixed with 0.2 cc. of tannic acid in concentrations of 0.06, 0.03, 0.015, 0.008, 0.004 and 0.002 percent. The tubes were then placed in a 37°C. water bath for 30 minutes, following which sensitized sheep cells, consisting of two parts of 2 percent cells and one part of rabbit amboceptor (4 units), were added to each tube in 0.6 cc. amounts. The tubes were reincubated for 30 minutes at 37°C., and the degree of hemolysis recorded.

The results are shown in Table X. It will be seen that tannic acid in the concentrations used was only slightly anticomplementary for human complement. This anticomplementary effect did not appear sufficiently strong to account for the complete blocking of hemolysis when tannic acid, complement, and red cells were mixed simultaneously. In contrast, tannic acid was markedly anticomplementary for guinea pig complement, causing inhibition of hemolysis in concentrations as low as 0.004 percent. The latter observation may explain the failure of guinea pig serum to produce hemolysis of red cells sensitized by tannic acid, as noted above.

In order to determine whether human serum was capable of preventing the adsorption of tannic acid by red cells, the following experiment was performed.

Two parts of a suspension of red cells were mixed with one part of varying dilutions of human serum, and one part of tannic acid solution was then added. After standing for 30 minutes, portions of each mixture were tested for hemolysis by the addition of complement. The results of an illustrative experiment are shown in Table XI. It will be seen that sensitization of the cells was prevented by human serum in a dilution of 1:100 and partially inhibited by a dilution of 1:500. An inhibiting effect of similar degree was demonstrated in

TABLE X

*The Anticomplementary Effect of Tannic Acid for Human and Guinea-Pig Complement, When Tested with Amboceptor-sensitized Sheep Cells*

| CONCENTRATION OF TANNIC ACID | VOLUME OF HUMAN COMPLEMENT (CC.) | | | | | |
|---|---|---|---|---|---|---|
| | 0.06 | 0.05 | 0.04 | 0.03 | 0.02 | 0.01 |
| *percent* | | | | | | |
| 0.06 | ++++ | ++ | 0 | 0 | 0 | 0 |
| 0.03 | ++++ | ++++ | ++++ | ++ | 0 | 0 |
| 0.015 | ++++ | ++++ | ++++ | ++++ | + | 0 |
| 0.00 | ++++ | ++++ | ++++ | ++++ | +++ | 0 |
| | VOLUME OF GUINEA-PIG COMPLEMENT (CC.) | | | | | |
| | 0.014 | 0.012 | 0.010 | 0.008 | 0.006 | 0.004 |
| 0.06 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.015 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.008 | +++ | + | 0 | 0 | 0 | 0 |
| 0.004 | ++++ | ++++ | +++ | + | 0 | 0 |
| 0.002 | ++++ | ++++ | +++∓ | ++ | + | 0 |
| 0.00 | ++++ | ++++ | ++++ | +++ | ++ | 0 |

serum which had been heated at 72°C. for 30 minutes, and also in a 0.1 percent solution of crystalline egg albumin.

The red cells contained in mixtures of tannic acid and serum were removed by centrifugation, washed, resuspended in NaCl solution, and tested with complement. Hemolysis of these cells did not occur. The concentration of tannic acid in the supernatant fluid after removal of the cells, determined colorimetrically, remained the same as before exposure of the cells.

The foregoing observations demonstrated that the adsorption of

tannic acid by red cells was blocked by small amounts of serum. It is probable that this effect, rather than an anticomplementary action by tannic acid, explains the failure of hemolysis to occur when cells, complement, and tannic acid were mixed simultaneously. The mechanism which underlies this blocking effect has not been determined, but it seems probable that tannic acid may form a union with a component of serum and thus be withheld from adsorption by the cells.

TABLE XI

*The Inhibitory Effect of Human Serum on the Sensitization of Red Cells by 0.06 Percent Tannic Acid, When Serum and Cells Were Combined before the Addition of Tannic Acid*

| SERUM DILUTION* | VOLUME OF COMPLEMENT (CC.) | |
|---|---|---|
| | 0.1 | 0.04 |
| 1:10 | 0 | 0 |
| 1:100 | 0 | 0 |
| 1:200 | ++ | 0 |
| 1:500 | +++ | ++ |
| 1:1000 | ++++ | ++++ |
| No serum | ++++ | ++++ |

* Figures refer to dilution of serum mixed with red cells before the addition of tannic acid.

*Attempts to Demonstrate the Adsorption of Complement by Sensitized Cells*

It has been shown that tannic acid may possess a dual affinity for the two participants in the hemolytic reaction, the red cell and the serum. It is adsorbed by the red cell and withheld from adsorption in the presence of serum. The latter effect is not specific for complement, since it is also a property of heated serum and egg albumin. It seemed possible that the action of tannic acid might be the result of its dual attraction for complement or a component of complement, and red cell. Therefore, the following experiments were undertaken in order to determine whether red cells which had been sensitized with tannic acid were capable of adsorbing complement.

20 cc. of a one percent suspension of red cells were mixed with 10 cc. of 0.06 percent tannic acid and allowed to stand for 30 minutes. The cells were then removed by centrifugation and taken up in one

cc. of chilled human serum. The mixture was kept in an ice bath for 18 hours, centrifuged in the cold, and the supernate compared for complement activity with an untreated sample of the same serum and with a sample mixed with unsensitized cells.

No decrease in the activity of complement after exposure to sensitized cells could be demonstrated, indicating that adsorption of complement by the sensitized cells did not take place to a degree detectable by this method.

The effect of exposing sensitized cells to complement in the cold on their susceptibility to hemolysis was studied.

A suspension of sensitized cells was prepared as in the preceding experiment and chilled in an ice bath. Aliquots of 0.6 cc. were mixed with 0.2 cc. of chilled complement diluted 1:2, and the mixture kept in an ice bath for one hour. The cells were then separated by centrifugation in the cold, and resuspended in 1.2 cc. of 0.7 percent NaCl. As long as these suspensions were kept cold, lysis did not occur. When they were warmed to 37°C., however, complete hemolysis took place within 10 minutes. Sensitized cells which were treated in a similar fashion with heat-inactivated complement did not hemolyze.

This observation indicated that the sensitized cells had either adsorbed a sufficient amount of complement to cause hemolysis when they were warmed, or that there remained enough complement in the fluid surrounding the sedimented cells to cause hemolysis. In order to settle this point, the effect of washing sensitized cells following exposure to complement was studied.

Sensitized cells were treated with complement in the cold as in the preceding experiment, centrifuged, and washed two times in cold NaCl solution. They were suspended in NaCl solution and placed in a 37°C. water-bath for 30 minutes. Hemolysis did not occur. These cell suspensions were then tested for their susceptibility to complement and compared with sensitized cells which had been treated in the cold either with NaCl solution alone or with serum heated at 56°C. for 30 minutes. Falling dilutions of complement were added, in 0.2 cc. amounts, to 1.0 cc. of cell suspensions, and the tubes placed in the water-bath for 30 minutes.

The results of this experiment are shown in Table XII, in which the

endpoint of complement activity is indicated in terms of the final dilution of complement in each tube. It will be seen that the sensitized cells which had previously been treated with cold complement were much more susceptible to the action of complement than cells treated with NaCl solution or heated serum. For washed sensitized cells alone, the end point of complement activity was in a final dilution of 1:240. After being exposed to cold complement and then washed, these cells were hemolyzed by dilutions of complement as high as 1:1920. No hemolysis was produced by serum which had been heated at 56°C. for 30 minutes.

TABLE XII

*The Increased Susceptibility of Human Red Cells to Lysis by Complement, When the Cells Were Sensitized with 0.06 Percent Tannic Acid, Treated with Complement in the Cold, Washed, and Then Tested with Complement at 37°C.*

| CELLS* | VOLUME OF COMPLEMENT (CC.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.02 | 0.01 | 0.005 | 0.002 | 0.001 | 0.0005 | 0.0001 | 0 |
| Treated with cold complement | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 |
| Untreated | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 |

* See text for method of treatment.

In an attempt to find an explanation for the heightened susceptibility of these cells, the effects of the two components of complement i.e., "mid-piece" and "end-piece," were studied. Fresh human serum was fractionated by the $CO_2$ method described by Ecker and Pillemer (9), and each component was made up in 0.7 percent NaCl. Sensitized cells which had previously been exposed to cold complement and washed, as in the preceding experiment, were tested with varying dilutions of mid-piece and end-piece. It was found that complete hemolysis was produced to an equal degree by each component alone, in final dilutions which were the approximate equivalent of a 1:120 dilution of whole serum. Neither mid-piece or end-piece, acting alone, were capable of causing hemolysis in sensitized cells which had not been treated with cold complement, although a combination of these components produced complete hemolysis.

## DISCUSSION

It has been shown that tannic acid has an effect on human erythrocytes which is, in its end-result, analogous to the action of a specific antibody. Red cells which are exposed to tannic acid become susceptible to lysis by complement from the same or other human individuals. By this method it is possible to perform titrations of human complement with endpoints similar to those obtained when complement is determined in the usual manner with amboceptor-sensitized sheep cells. Moreover, as shown in another communication (7), this hemolytic system can be employed as the indicator in certain types of complement fixation tests.

Certain conditions are necessary before the hemolytic reaction with tannic acid can be elicited with reproducible results. The concentration of sodium chloride is of the first importance. The reaction may be partially or even completely inhibited if physiological saline solution is used as the diluting fluid for the reagents, and it is necessary to use less concentrated salt solutions. In the present study the optimal sodium chloride concentration was found to be 0.7 percent. Higher concentrations inhibit the hemolytic reaction in two ways: tannic acid is prevented from preparing the red cells for lysis, and complement is prevented from lysing cells which have already been prepared with tannic acid. It is of interest that hemolysis occurs when isotonic glucose is substituted for sodium chloride in the diluting fluid, although complement is less effective in glucose than in 0.7 percent sodium chloride.

The order of mixing the reagents employed in the reaction is also of importance. It has been shown that the red cells and tannic acid must be placed together first, and a period of at least 5 minutes allowed for sensitization of the cells before the addition of complement. If complement is present at the time when cells and tannic acid are combined, hemolysis does not occur. Relatively small amounts of fresh or heated serum, or albumin solution, prevent the sensitization of red cells by tannic acid. The probable explanation for this effect is that the tannic acid combines with the soluble protein and is thus withheld from adsorption by the cell.

The optimal concentration of tannic acid was found to lie between 0.12 and 0.015 percent. Within this narrow range, the time required

for the sensitization of red cells varies inversely with the concentration of tannic acid. The optimal concentration of red cells is a one percent suspension of washed cells. The optimal pH for sensitization of red cells is between 7.0 and 8.0.

That the factor in human serum which caused hemolysis is actually complement is indicated by the following findings: the factor is completely inactivated by heating at 56°C. for 30 minutes, and by two known anticomplementary agents, heparin and congo red. It is fixed in the presence of antigen-antibody combinations, as has been shown in another paper (7). Finally, when human serum is tested for complement activity simultaneously with tannic-acid-sensitized human cells and amboceptor-sensitized sheep cells, the endpoints are identical.

The mechanism of action of tanic acid is not known. The effects of this substance on tissues in general have been attributed to its capacity to precipitate soluble proteins, to its dehydrating property, or to its ability to unite chemically with proteins. Olitsky and Cox (10) have shown that the intranasal instillation of tannic acid in mice causes an increased resistance to intranasally introduced equine encephalitis virus, which was attributed to a direct effect of tannic acid on the nasal mucosa. Recently, Green (11) has demonstrated that tannic acid has the property of inhibiting influenza virus *in vitro* and *in vivo*, presumably due to an action of this substance on the virus itself.

The results of the present study indicate that tannic acid may combine with some component of the red cell. When this union has been established, the cells may be washed repeatedly without loss of their susceptibility to complement. Moreover, when the red cells are added to tannic acid solution and then removed by centrifugation, the solution loses some of its capacity to sensitize new cells and the concentration of tannic acid in the solution, as determined colorimetrically, becomes decreased. These observations suggest that tannic acid is adsorbed to the red cells.

Once tannic acid has united with the red cell, it may act as a source of attraction for complement or for some component of complement, thus making it possible for the latter to be brought to the cell surface. An actual adsorption of complement by sensitized cells has not been

demonstrated, but suggestive evidence for this was offered by experiments in which it was shown that when sensitized cells were mixed with complement in the cold and then removed from complement, washed, and resuspended in sodium chloride solution, they were susceptible to a much smaller amount of complement than is ordinarily required for the hemolysis of sensitized cells. Moreover, these cells were lysed when either mid-piece or end-piece were added alone, although neither of these components was capable by itself of lysing sensitized cells which had not been treated with complement. Heat-inactivated serum had no effect on these treated cells. These results suggest that during the time of exposure of sensitized cells to complement in the cold, some portion of complement may have been taken on the cell, so that subsequently the addition of a very small amount of complement was sufficient to cause hemolysis. No explanation can be given for the capacity of both mid-piece and end-piece to bring about hemolysis in this case, although it is possible that each of the fractionated components may have contained residual traces of the ether.

An alternative explanation for the heightened susceptibility of sensitized cells following treatment with complement in the cold is that the cells were actually injured in some fashion during the period of exposure to complement, although this injury was not sufficient to cause hemolysis. Subsequent exposure of the cells to complement at 37° C. might then have resulted in hemolysis with smaller amounts of complement than are usually necessary.

It is of interest that the hemolytic reaction with tannic acid and complement could be elicited reproducibly only with the homologous system involving human cells and serum. Attempts to produce hemolysis with cells and complement from rabbit, guinea pig, sheep and mouse sources were unsuccessful. In the case of the guinea pig, it has been shown that the failure may have been due to a primary anticomplementary effect of tannic acid on guinea pig complement. Attempts to demonstrate the reaction between cells from one species and complement from another yielded essentially negative results. Hemolysis sometimes occurred, but was usually incomplete and required high concentrations of complement. It is not known why human cells and complement should provide the most favorable system for the study of this phenomenon.

The results of this study do not provide information which bears directly on the problem of the mode of action of complement, but the method may be of use in an approach to this problem. The double affinity of tannic acid for the red cell and for serum protein suggests the possibility that this substance may act by bringing complement (or a component of complement) into close relationship with the red cell surface. It has been suggested by previous workers (12) that the hemolytic action of complement may be analogous to that of a surface-active agent. Further studies on this possibility are in progress.

## SUMMARY

1. Treatment of human red cells with tannic acid rendered them susceptible to hemolysis by fresh human serum from the same or other individuals. Demonstration of this reaction depended upon the presence of optimal concentrations of sodium chloride (0.7 percent), tannic acid (0.12 to 0.015 percent), and red cells (one percent). The optimal pH range lay between 7.0 and 8.0.

2. This reaction could not be demonstrated with cells and serum from rabbit, guinea pig, mouse or sheep sources.

3. It was necessary to allow the cells and tannic acid to stand together for at least five minutes before the cells became completely sensitized. Longer periods were required when lower concentrations of tannic acid were employed.

4. When titrations of human complement were performed simultaneously with tannic acid-sensitized human red cells and amboceptor-sensitized sheep cells, the endpoints of complement activity were identical.

5. The hemolytic factor in human serum was inactivated by heating at 56°C., and by the addition of heparin or congo red.

6. Sensitization of red cells by tannic acid failed to occur if serum was present at the time when cells and tannic acid were mixed.

7. Red cells which were sensitized by tannic acid could be washed without losing their susceptibility to hemolysis by complement. Exposure of a tannic acid solution to red cells resulted in a diminution in the capacity of this solution to sensitize new red cells, as well as in a decrease in the concentration of tannic acid estimated colorimetrically.

8. When tannic acid-sensitized cells were exposed to complement in

the cold, and then washed, they became susceptible to hemolysis by much smaller quantities of complement. Such cells were also hemolyzed by complement mid-piece or end-piece alone. They did not lyse spontaneously, nor in the presence of heat-inactivated complement.

## REFERENCES

1. Landsteiner, K.: The Specificity of Serological Reactions. Harvard University Press, 1946.
2. Reiner, von L., and Fisher, O.: Ztschr. f. Immunitätsforschung, 1929, **61**, 317.
3. Neufeld, F., and Etinger-Tulczynska, R.: Zbl. Bakt., I Abt., Orig., 1929, **114**, 252.
4. Freund, J.: Proc. Soc. Exper. Biol. and Med., 1929, **26**, 876.
5. Gordon, J., and Thompson, F. C.: Brit. J. Exper. Path., 1936, **17**, 159.
6. Gordon, J., and Thompson, F. C.: Brit. J. Exper. Path., 1937, **18**, 390.
7. Thomas, L., and Peck, J. L.: Proc. Soc. Exper. Biol. and Med., 1948, **67**, 475.
8. Greissmayer, L.: Zeitschr. anal. chem., 1872, **11**, 43.
9. Ecker, E. E., and Pillemer, L.: Ann. N. Y. Acad. Science, 1942, **43**, 63.
10. Olitsky, P. K., and Cox, H. R.: Science, 1934, **80**, 566.
11. Green, R. H.: Proc. Soc. Exper. Biol. and Med., 1948, **67**, 483.
12. Osborn, T. W. B.: Complement or Alexin. Oxford University Press, 1937.

# A SIMPLE VOLUMETER FOR MEASURING THE OXYGEN CONSUMPTION OF SMALL ANIMALS[1]

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Received for publication August 13, 1948

Although a variety of methods exist for measurement of oxygen consumption of small animals (1, 2), the apparatus to be described was designed to meet the requirements of simplicity of construction, availability of materials, accurary and speed of operation. The principle, which reflects the 100-year-old method of Regnault and Reiset (6), depends upon measurement of the rate of change in volume in a closed-circuit metabolism chamber at constant temperature and pressure. The innovation of the present method is that volume change is determined by means of a coiled volumeter burette which is maintained at constant temperature in the bath that houses the metabolism chamber.

## APPARATUS

A conventional *Warburg-Barcroft constant-temperature bath* with stirrer is employed with a thermoregulator sensitive to 0.05°C. and set to 28.0°C.

In one end of the bath is seated the *metabolism chamber*, a rectangular brass box, open at the top, 28 cm. long $x$ 20 cm. wide $x$ 13 cm. deep (Figure 1). The box is supported on legs to permit circulation of bath water over all six faces, and the underside is weighted with about 9 kg. of lead plate to prevent flotation. A short brass tube, 1 cm. internal diameter, pierces one long side of the box. The lid of the box is a Lucite plate, 30 $x$ 30 $x$ 1 cm., with a 5 mm.-deep milled slot on the under surface to permit an air-tight petrolatum seal when fitted in place. Since the Lucite sheet is larger than the base of the box, a projecting

[1] Work done under a contract between the Office of Naval Research, U. S. Navy Department and the Johns Hopkins University.

shelf is available for support of the volumeter to be described presently. The Lucite is secured to the box by five steel springs distributed on three sides. In the Lucite are bored three holes, 12 mm. in diameter, tapered to fit No. 1 rubber stoppers and placed as indicated in the figure. These holes permit passage into the chamber of a stirrer, a thermometer and an outlet tube with a glass stopcock.

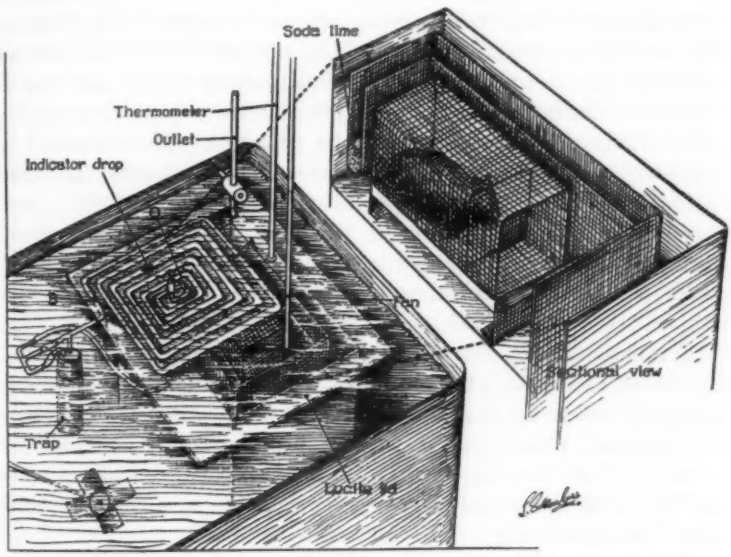


FIG. 1

The chamber contains soda lime, a humidifier, and the animal cage. *Soda lime* is contained in a U-shaped trough of No. 8 mesh metal screening which lines three sides of the box and embraces the animal cage. On the floor of the box, beneath the animal cage is placed a watch glass containing several grams of *anhydrous calcium chloride* granules moistened with a little water. This agent maintains chamber humidity at about 30 per cent (5). The *animal cage* is of No. 8 mesh wire screen with a false floor of No. 2 mesh screen, providing maximum animal space of 7 $x$ 18 $x$ 5 cm. Effective length of the cage can be shortened by a sliding end wall, secured by a wooden pin. Below the

false floor is a removable brass tray, lined with filter paper, moistened with approximately 0.01 N HCl to neutralise urinary ammonia.

Placed flat on the Lucite shelf is the *coil volumeter* made of 4 mm. internal diameter Pyrex glass tubing, silicone-lined (Drifilm, #9987, General Electric Co.) to prevent dispersion of the indicator drop. The tubing is approximately 3 m. long, contains 7 coils, and in its outer dimensions approximates a square 20 cm. wide. The central end of the volumeter curves to a perpendicular with respect to the plane of the coil and serves as an air inlet. The volumeter tube terminates as a T-tube; one arm is connected by rubber tubing to the brass tube in the side of the metabolism chamber; the other arm leads to a trap for the volumeter indicating fluid, to be described. The volumeter is kept in place by four sets of pins screwed into the Lucite top. The volumeter is marked at three points and calibrated. At 28°C., bath temperature, the volume between the initial point, O, and the intermediate point, A, is of the order of 30 ml.; between O and the terminal point, B, 35 ml. These volumes were selected to provide metabolism periods of some five to 10 minutes for normal rats weighing about 200 g.

Circulation within the chamber is maintained by a fan (1 $x$ 5 cm.) driven at 1200 r.p.m. by an electric stirring motor, mounted independently above the chamber. The shaft of the stirring fan, made of stainless steel, passes into the chamber through a 5 cm. long, smoothly machined brass bushing seated in a rubber stopper. This seal does not leak under pressures exceeding 10 mm. Hg. Under these conditions the chamber temperature approximates 29.0°C.

The chamber is illuminated by a fluorescent lamp suspended approximately 18 inches above the bath to prevent local heating.

## TECHNIC AND RESULTS

The animal is placed in the cage; the cage size adjusted to confine it in a comfortable space which just permits turning. Soda lime, moistened calcium chloride and the animal cage are placed within the chamber. The Lucite lid, greased appropriately, is set in place and fixed with springs. The volumeter is attached. The thermometer, the outlet tube and the stirrer are assembled.

With the outlet tube open, the chamber is gassed with oxygen through the open end of the volumeter. It is desired only to obtain

an atmosphere sufficiently rich in oxygen to permit observation of the animal for several hours without the supervention of anoxia. After appropriate gassing, the outlet tube is closed and the bath is filled with water to a level about 2 cm. above the chamber lid. The graduated portion of the volumeter is, therefore, submerged; only the air inlet is above water.

At least 30 minutes are allowed for thermal equilibration and to permit the animal to fall asleep. Then a 'drop' of water, approximately 0.15 ml., colored with methylene blue, is introduced into the inlet of the volumeter to seal the circuit. Carbon dioxide evolved by the animal is absorbed by soda lime. Pressure within the chamber decreases, therefore, as oxygen is consumed. *Since the barrier between atmospheric and chamber pressures is freely movable, continuous equilibration to barometric pressure occurs; the 'drop' flows in the volumeter toward the chamber.* The time occupied by the 'drop' in passing between the graduations of the volumeter (i.e., the rate of decrease in chamber volume) is timed with a stopwatch. From this measurement is calculated the rate of oxygen consumption of the animal.

Since the animal is under observation through the Lucite top, it is possible to exclude observations obtained during and after periods of activity. Furthermore, by reading the chamber thermometer at the beginning and end of each run, it is possible to use only those periods during which temperature is constant.

Under these operating conditions the rat usually falls asleep within 20 minutes. When equilibration is complete, as signalled by constancy of the chamber temperature, readings are begun. The elapsed times for a 'drop' indicator to move from O to A and to B are measured by a stopwatch. This constitutes an internal check on the measurements. In 99 such measurements the mean difference between A and B readings was 0.02 ml. $O_2$/min./100 g. rat (maximum difference: 0.08; standard deviation: 0.0185). At least three such complete measurements are made during periods of sleep, and the mean of these values is taken as the "basal" oxygen consumption for the experiment. Under usual circumstances a complete determination requires from 60 to 90 minutes.

As tests of reliability of the apparatus, 33 such complete determinations of oxygen consumption were performed on a litter of eight male rats of mixed albino-Whelan strain, approximately 90 days old and

200 to 250 g. in weight. All determinations were performed after a fast of 17 to 22 hours.

Triplicate determinations (i.e., three separate runs through the coil during a single residence in the metabolism chamber) agreed with a mean deviation of ±3.16 per cent (range: 0 to 10.2 per cent; S. D.: ±2.6 per cent). The average of triplicate determinations was recorded as the "basal" oxygen consumption. This value was calculated on at least three occasions for each animal, and the arithmetic average of these measurements was recorded as the mean "basal" oxygen consumption. In the series of eight litter-mates, the mean basal oxygen consumption was 1.83 ml. $O_2$ STPD/min./100 g. rat (Table 1).

TABLE 1

*$O_2$ Consumption of 8 Sibling Male Rats*

All values expressed as ml. $O_2$/min./100 g. body weight

| | RAT | | | | | | | | GROUP |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| No. of Determinations | 4 | 3 | 4 | 6 | 5 | 3 | 3 | 5 | 33 |
| Maximum | 1.87 | 1.84 | 1.95 | 1.89 | 2.03 | 1.99 | 1.85 | 2.06 | 2.06 |
| Minimum | 1.76 | 1.77 | 1.67 | 1.67 | 1.75 | 1.85 | 1.76 | 1.67 | 1.67 |
| Mean | 1.82 | 1.80 | 1.83 | 1.78 | 1.87 | 1.90 | 1.80 | 1.83 | 1.83 |
| S.D. | | | | | | | | | ±0.036 |

## CALCULATION

It is assumed that gas within the volumeter is saturated with water vapor, due to the advancing 'drop', and that its temperature is that of the water bath. On the other hand, gas within the chamber is unsaturated and may be warmer, due to imperfect dissipation of animal heat to the bath. However, given the chamber temperature and vapor pressure, it is possible to convert the measured volume in the volumeter to the actual volume of oxygen consumed by the animal in the chamber. Finally, this volume may be expressed in terms of standard temperature and pressure, dry.

Chamber aqueous vapor pressure is assumed from the following considerations. Vapor pressure above saturated $CaCl_2 \cdot 6H_2O$ at

24–25°C. is given as 7.15 mm. (5). It is assumed that it is 8 mm. at 28° to 30°. Since the chamber contains excess soda lime with very low vapor pressure, chamber vapor pressure is somewhere between 0 and 8 mm. Because moistened filter paper does not dry appreciably within the chamber it is suspected that chamber vapor pressure is nearer 8 than 0 mm. and is assumed, therefore, to be 6 mm. Sample calculations indicate that an error of 4 mm. in estimating the aqueous vapor pressure will introduce an error of less than 0.5 per cent in the final calculation.

The volume of oxygen consumed, $V_1$ (STPD), is calculated from the calibrated volumeter, V, by applying the equation (3):

$$V_1 = V\left(\frac{1}{1 + 0.00367t}\right)\left(\frac{P - e}{760}\right)$$

where t = chamber temperature (°C.); P = corrected barometric pressure (mm. Hg); and e = aqueous vapor pressure = 6 mm. Hg.

The rate of oxygen consumption is calculated by dividing $V_1$ (ml.) by the time (min.) required for its consumption. Finally, the rate of oxygen consumption is expressed with reference to body weight (ml. $O_2$/min./100 g. rat).

## SOURCES OF ERROR

The validity of the observations depends upon maintenance of constant pressure and temperature.

Changes in barometric pressure during the traverse of the indicator 'drop' through the volumeter (5–10 min.) are insignificant under usual laboratory conditions, for a 5 mm. variation in barometric pressure alters the apparent volume by less than one per cent. It was assumed that the system was frictionless, and that the flow of the indicator 'drop' did not hinder instantaneous equilibration of atmospheric and chamber pressures; for pressure differentials of less than 2 mm. of water produced immediate movement of the indicator 'drop'. The reliability of these assumptions was supported by close agreement between rates of oxygen consumption calculated from the speed of the same 'drop' traveling along the volumeter from O to A and from O to B.

With respect to temperature, it can be calculated that, in the apparatus described, a change in chamber temperature of 0.05°C. would

produce an error of about 5 per cent, due to thermal alteration of chamber gas pressure. By using a precision chamber thermometer which was sensitive to changes of less than 0.05°C., it was possible to eliminate the occasional period in which fluctuations in chamber temperature exceeded that value.

Rate of absorption of carbon dioxide by soda lime is a function of the circulation rate of gas within the chamber. Morrison has developed an expression which describes this relationship (4) and has pointed out that a circulation rate of 1.0 per minute requires a metabolism period of no more than five minutes in order that delay in the absorption of carbon dioxide by the soda lime not introduce a lag error. By the introduction of smoke and of carbon dioxide into the chamber, and observation of movement and rate of uptake, respectively, it was estimated that the circulation rate in the chamber described here exceeds 10. Despite this rapid movement of air the rats appear comfortable and sleep.

## SUMMARY

1) A closed-circuit, constant temperature, constant pressure apparatus is described for measurement of oxygen consumption of small animals. It offers simplicity of construction from readily available materials and speed of operation.

2) Triplicate determinations of basal oxygen consumption in the rat agreed within ±3.2 per cent.

## REFERENCES

1. Benedict, F. G. and Macleod, G.: The heat production of the albino rat. I. Technique, activity control and influence of fasting. J. Nutr., 1929, **1:** 343.
2. Benedict, F. G.: A multiple chamber respiration apparatus for rats and other small animals. J. Nutr., 1930, **3:** 161.
3. Carpenter, T. M.: Tables, factors and formulas for computing respiratory exchange and biological transformations of energy. (3rd Ed.) Carnegie Inst. Washington, Washington, D. C., 1939.
4. Morrison, P. R.: An automatic apparatus for the determination of oxygen consumption. J. biol. Chem., 1947, **169:** 667.
5. Obermiller, J.: Die Einstellung von Luft auf bestimmte Trocknungs oder Feuchtungsgrade mit Hilfe von Salzen und ähnlichen Stoffen. Z. physik. Chem., 1924, **109:** 145.
6. Regnault, V. and Reiset, J.: Récherches chimiques sur la respiration des animaux des diverses classes. Ann. chim. et d. Physique, 1849, **26:** 299.

# THE CLINICAL RESULTS OF COMBINED PENICILLIN AND ICE THERAPY*

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Received for publication August 14, 1948

Smith (1) showed that infection of an extremity could be treated successfully by chilling the affected part for a prolonged period.

Thompson and Trumpter (2), while searching for a method to prolong the blood level of a single injection of penicillin, found that ice applied to the area injected caused slow absorption of the drug into the blood stream.

The efficacy of penicillin in the treatment of certain infections is so well established that any mention of its history is unnecessary.

It seemed reasonable to presume that, if an infected area was chilled and then locally injected with penicillin, a high concentration of penicillin could be localized at the site of infection for a long period of time. It also seemed possible that an infected extremity could be treated more efficaciously by a combination of cold and penicillin than by penicillin or ice alone. With this in mind, the following group of experiments were initiated.

## EXPERIMENT I

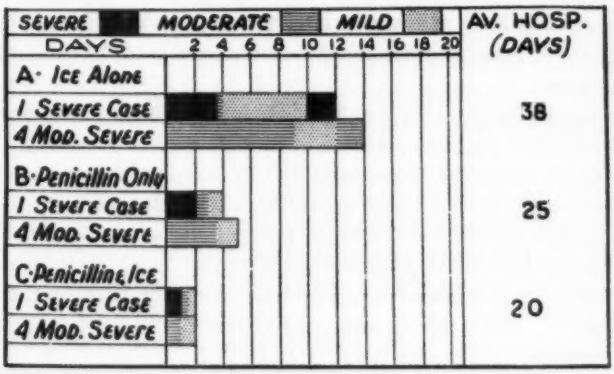
Includes 15 cases of secondarily infected varicose ulcers of the lower extremity. These 15 cases were divided into three groups, designated I *a*, I *b*, and I *c*, respectively. Each group contained one severe and four moderately severe ulcers. In each case, the ulcer had persisted for at least three months, and had been treated for at least one month with such general measures as bed rest and elevation of the affected extremity, supplemented by local treatment at the site of the ulcer with hydrogen peroxide and 5% sulfadiazine ointment. In all cases cultures showed the presence of *beta hemolytic Streptococcus and hemolytic Staphylococcus aureus, in addition to other organisms.*

* Appreciation is expressed to M. M. Wintrobe, M.D., for his suggestions in regard to the manner of presentation of this manuscript.

*Procedure and Results*

Group I *a*. The 5 patients in this group were treated by wrapping the extremity in two face towels and placing five ice bags on the leg for a period of five days.

In the severe case of this group, pain immediately disappeared and after 3.5 days of treatment, evidence of secondary infection slowly subsided. The ice bags were removed at the end of five days. However, on the tenth day a recrudescence of symptoms occurred and penicillin therapy was necessary to complete cure.




EXPERIMENT I. 15 CASES OF VARICOSE ULCERS WITH SECONDARY INFECTION

The 4 moderately severe cases were treated in the same manner as the above. In these cases, however, the infection became minimal, on the average, on the ninth day and a recrudescence of symptoms appeared on the twelfth. Penicillin therapy was then instituted as in the above.

Patients of Group I *a* were discharged when epithelialization of the ulcer was complete and patients were without symptoms. *Average hospitalization after ice therapy was initiated was thirty-eight days.* NOTE, however, that cure was not complete without additional penicillin therapy.

Group I *b*. The 5 patients in this group received penicillin intragluteally—50,000 units, every three hours for a total of 1.2 to 1.6 million units.

In the severe case of this group, all manifestations of secondary infection had disappeared by the end of the fourth day.[1]

In the 4 moderately severe cases all manifestations of secondary infection had vanished within 3.5 to 6 days—5 days on the average.

There was no recrudescence of secondary infection in patients of Group I *b*. Again the patients were discharged after the ulcer was epithelialized and the patients without symptoms. *Average hospitalization after therapy was initiated was twenty-five days.*

Group I *c*. In the 5 cases of this group, ice was placed about the site of the ulcer for one hour. The area was then prepared with tincture of merthiolate, and 500,000 units of penicillin, dissolved in 10 cc. of 1% novocain, was infiltrated about the site of the ulcer, and five ice bags were kept about the ulcer for a period of 5 days.

In the severe case in this group, manifestation of secondary infection had disappeared by the end of the second day.

Of the 4 cases of moderate secondary infection, all manifestations of secondary infection in 2 cases, had disappeared before the end of the second day and in 2 at the end of the first day.

There was no recrudescence of infection in this group and *average hospitalization after therapy was initiated was 20 days.*

## EXPERIMENT II

Forty-five cases of secondarily infected epidermophytosis were divided into three groups, designated II *a*, II *b*, and II *c*, respectively. Each group contained 3 patients with severe secondary infection. Infecting organisms, streptococci and staphylococci. No cases in which the infecting organism was a gram negative bacillus were considered.

### *Procedure and Results*

Group II *a*. Fifteen cases were treated by wrapping the infected extremity in two face towels and placing ice bags on the leg for twelve hours.

In the 3 severe cases of this group all manifestations of infection had disappeared by the end of the sixth day.

In the 7 moderately severe cases, all manifestations of infection had likewise disappeared by the end of the sixth day.

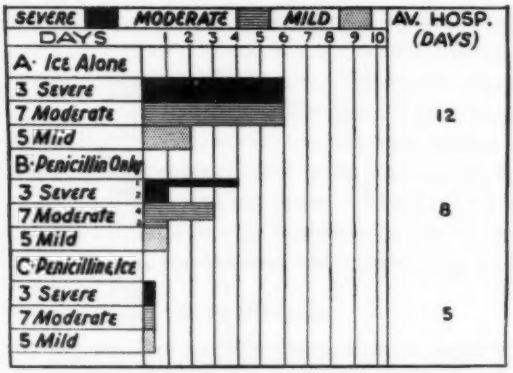
[1] Such as fever, redness, swelling, and edema.

In the 5 mild cases, the infection was cleared up at the end of the second day. *Average hospitalization, 12 days.*

Group II *b*. The fifteen patients of this group received 30,000 units of penicillin intragluteally, q.3 h for a total of 240,000 units.

In the 3 severe cases of infection in this group, 1 patient became asymptomatic at the end of the fourth day of treatment, while the remaining two became asymptomatic at the end of 24 hours.

In the 7 patients with moderate infection, 4 became asymptomatic at the end of the third day, while the remaining 3 required only 24 hours to be entirely free of symptoms.




EXPERIMENT II. 45 CASES OF INFECTED EPIDERMOPHYTOSIS

Of the five mild cases, all were asymptomatic at the end of 24 hours. However, in this group, 4 of the 15 cases developed a recrudescence of symptoms as soon as the penicillin was stopped and it was necessary to complete cure. *Average hospitalization, 8 days.*

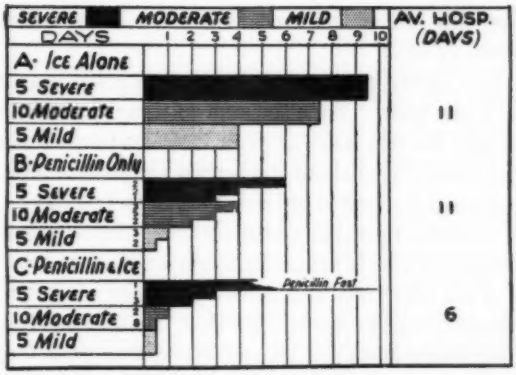
Group II *c*. The 15 patients of this group received local penicillin and ice treatment as described under I *c*.

All 15 cases of this group became asymptomatic within 12 hours and there was no additional treatment necessary in order to control secondary infection. *Average hospitalization, 5 days.*

## EXPERIMENT III

Sixty cases of wound infection of the extremities were divided into three groups containing twenty patients in each group, designated

III *a*, III *b*, and III *c*, respectively. Each group contained 5 patients with marked toxic symptoms, such as hyperpyrexia, marked redness and tenderness about the wound, and regional lymph node tenderness; ten patients showing a moderate febrile response and a moderate amount of tenderness and regional lymph node tenderness; and five patients showing only mild symptoms. The five cases in each group compared fairly well in regards to length, depth, and irregularity of cut surface, with the wounds of patients in each of the other groups. The




EXPERIMENT III. 60 CASES OF WOUND INFECTION OF EXTREMITIES

infecting organisms were usually streptococci. None of the infections were produced by gram negative bacilli.

*Procedure and Results*

Group III *a*. The twenty patients of this group received ice treatment over a period of five days.

In the five severe cases, manifestations of infection disappeared within a period of nine to ten days.

In the 10 moderately severe cases, manifestations of infection disappeared within seven to eight days.

In the five mild cases, all signs of infection cleared up within three to five days. *Average hospitalization, 11 days.*

Group III *b*. The twenty patients in this group received intragluteally, 50,000 units of penicillin every three hours until symptoms of

infection had remained absent for twenty-four hours. Total dosage, 500,000 to 2.5 million units.

In the five severe cases of this group, infection disappeared at the end of the sixth day in two cases, at the end of the fourth day in two cases, at the end of the third day, in one case.

In the ten cases of moderately severe infections, 3 cases were asymptomatic by the end of the fourth day, five by the end of the third day, and two by the end of the second day.

In the five mild cases, three were asymptomatic by the end of the first day, and two, twelve hours after initiation of treatment. *Average hospitalization, 11 days.*

Group III *c*. In the twenty cases in this group, ice was placed about the wound for one hour. Then the area was prepared with tincture of merthiolate and 500,000 units of penicillin, dissolved in 10 cc. of 1% novocain, were infiltrated about the wound and five ice bags were kept about the wound for a period of twelve hours to two days.

One case of severe infection was apparently penicillin fast. Among the four remaining cases of severe infection, one became asymptomatic by the end of the third day and three by the end of the second day.

Of the ten cases with moderate infection, two became asymptomatic in twenty-four hours and eight became asymptomatic in twelve hours. In the five mild cases, all were asymptomatic twelve hours after initiation of treatment. *Average hospitalization, excluding one which did not respond to treatment, was six days.*

## DISCUSSION

In the series of cases presented, ice-penicillin therapy proved decidedly superior therapeutically to either interrupted intragluteal penicillin or ice. The advantages are enumerated as follows:

1. Hospitalization decidedly less.
2. More rapid disappearance of the signs and symptoms of infection.
3. Lower total dosage of penicillin to effect a cure.
4. Disappearance of pain on chilling. (Pain also vanishes with ice alone.)

The reason for such excellent results is a matter of interesting conjecture. The mechanism of the action of penicillin is unknown, but it has been suggested by Chain and Duthie (3) that penicillin is part of

an enzyme or activates an enzymatic system. Hobby (4) reported that the greatest destruction of organisms occurs during the phase of rapid multiplication. Chain and Duthie (3) lend additional creditability to Hobby's findings when they reported that during the resting of the culture cycle of the staphylococcus, large concentration of penicillin had no effect on the rate of oxygen uptake. During the earlier lag phase and the logarithmic phase of growth, penicillin exerted a strong inhibitory effect on oxygen uptake. It therefore seems likely that penicillin interferes with a metabolic function involved in the early stages of bacterial development. In view of the above, the clinical results of the ice-penicillin therapy seem paradoxical. Lee, *et al.* (5) found that if cultures of *S. aureus* are kept even as low as 14°C. until measurable growth has occurred in control tubes, a decrease in viable count is found in the ones containing penicillin. If the cultures were kept at 14°C. for some time the killing action of the penicillin is observed.

The crux can readily be explained when it is considered that Hobby's work was done in vitro, while our set of experiments were carried out in vivo. The entire modern concept of combating infection is ultimately based on the physiological inactivation of pathogenic organisms, so that eventually the body defenses can attack and overcome the invading organism. The cooling of the infected area in the presented cases is not sufficient to stop entirely the bacterial division, and, therefore, some of the beneficial killing effects of penicillin noticed by Hobby in vitro, *must be in action*. Assuredly the bacteria are inhibited by the chilling effect, and as a result it seems probable that they are more susceptible to body defenses, namely phagocytosis and the formed antibodies. In this respect it was shown by Chandler, *et al.* (6) that penicillin plus serum of persons who were infected with an organism was more effective in inhibiting the growth of that organism than penicillin plus serum of persons not infected.

As early as 1865 Cooke (7) recognized that ice checked the inflammation and suppuration of cancer which was secondarily infected. Smith (1) describes healing processes and production of new capillaries (where prolonged refrigeration has been employed in infected wounds) continued slowly, but satisfactorily in areas under constant refrigeration at 14°C.

There are additional possibilities of elucidating the reason for the excellent clinical results of the ice-penicillin therapy, namely, the growth-inhibiting properties of penicillin may be inactivated, or even destroyed, by substances of microbial origin. Abraham and Chain (8) found that *E. coli* contained an enzyme-like substance, penicillinase. Cavillito and Bailey (9) suggested that the main action of many antibiotic substances rests on their ability to interfere with the normal functions of sulfhydryl groups in bacterial metabolism. However, Frazier and Friden (10) suggest, "that the inhibition of antibiotics by sulfhydryl compounds may occur as the result of a chemical reaction involving the two substances and leading to the formation of an inactive product and does not require the hypothesis that the action of the inhibition is the restoration of normal equilibrium to an enzyme system involving SH compounds which produce a chemical reaction by which penicillin is inactivated"; the ice may well slow up any chemical reaction which tends to destroy the activity of penicillin.

Gerber *et al.* (11), suggested that when interrupted intragluteal penicillin is given, it is difficult to obtain a significant concentration of penicillin in many of the body tissues, and maintains that this is especially so in cases of subacute or chronic infections where the organisms are encased in dense granulomatous tissue. He also suggested injection of the penicillin directly into the infected site.

We have established that a significant level of penicillin may be maintained over a period of at least 31 hours at the site of a wound, when employing the ice-penicillin therapy.

Nine patients, with either a simple or severe wound of the lower extremity, were treated as follows:

Five ice bags were placed about the infected wound of the lower leg for one hour. The ice was removed and the dealbated area about the wound was prepared with tincture of merthiolate. One hundred thousand units of penicillin dissolved in one per cent novocain were infiltrated about the wound. Two face towels were placed about the area to act as an aegis to the superficial tissues and the five ice bags were replaced. Samples of blood were withdrawn from the cephalic vein at frequent intervals. The first sample was taken six hours, and the last sample 31 hours after the injection. In all cases penicillin could be demonstrated in the blood stream at the end of 31 hours. As long as

a penicillin blood level can be demonstrated, the tissue at the site of injection must still contain a high level of penicillin, for Rantz and Kirly (12) showed penicillin to be rapidly excreted in the urine and found no evidence of destruction of penicillin in the tissues.

When the ice-penicillin method of therapy is employed, not only is the penicillin kept in the local tissues for a long period of time, but the concentration of the penicillin at the site of infection must be much greater than that obtained when using the intragluteal method of administering the drug.

## SUMMARY

1. Fifteen cases of infected varicose ulcers, forty-five cases of secondarily infected epidermophytosis, and sixty cases of wound infection were divided into groups and treated with either ice, interrupted intragluteal penicillin or ice, plus local infiltration of penicillin.

2. The results of each type of therapy are compared in regards to the rapidity of disappearance of the signs and symptoms of infection, the total dosage of penicillin necessary to produce cure and the length of hospitalization.

3. A discussion of the possible reasons for the excellent results of the ice-penicillin therapy is given.

## BIBLIOGRAPHY

1. Smith, L. W.: Use of Cold in Medicine, "Ann. Ind. Med." **17:** 618, 1942.
2. Lt. Cmdr. Trumpter, Max, and Lt. Cmdr. Hutter, A. M.: Prolonging Effective Penicillin., "Action Science" Vol. 100: 432–434 (Nov. 10) 1944.
3. Chain, E., and Duthie, E. S.: "Bactericidal and Bacteriolytic Action of Penicillin of the Staphylococcus," Lancet **1:** 652 (May 26) 1945.
4. Hobby, Gladys L., Meyer, Karl and Chaffer, Eleanor: Observations on the Mechanism of Action of Penicillin, "Proc. Soc. Exper. Biol. and Med." **50:** 281 (June) 1942.
5. Lee, S. W., Foley, J. E. and Epstien, J. A.: Mode of Action of penicillin, "Journal of Bacteriology," Vol. 48, No. 4, (Oct.) 1944.
6. Chandler, Velma L., Price, Clefford W. and Randall, William A.: Control and Evaluation of Blood Serum Assays for Penicillin. "Science," (Oct. 5) 1945, Vol. 102, No. 2649, pp. 355–356.
7. Cooke, T. W.: "On Cancer, Its Allies and Counterfeits," London, 1865.
8. Abraham, E. P. and Chain, E.: An Enzyme from Bacteria Able to destroy Penicillin, "Nature" **146:** 837 (Dec. 28) 1940.

9. CAVALLITO, C. J. AND BAILEY, J. H.: Preliminary Note on the Inactivation of Antibiotica, "Science" **100:** 390 (Oct. 27) 1944.
10. FRAZIER, C. N. AND FRIENDEN, E. H.: "Action of Penicillin," (March 16) 1946. Vol. 130, No. 11: 677.
11. GERBER, I. E., SCHWARTZMAN, G. AND BAEHR, G.: Penetration of Penicillin into Foci of Infection, "J. A. M. A.," (March 23) 1946. Vol. 130, pp. 761–764.
12. RANTZ, LOWELL A., KIRBY, WILLIAM M. M., AND RANDALL, ELIZABETH: The Absorption and Excretion of Penicillin Following Continuous Intravenous and Subcutaneous Administration, Journal of Clinical Investigation, Vol. XXIII, pp. 789–794, (Sept.) 1944.

# CIRCULATING ANTICOAGULANTS: A TECHNIQUE FOR THEIR DETECTION AND CLINICAL STUDIES

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Received for publication August 14, 1948

Several reports (1, 2, 3, 4, 5, 6, 7, 8, 9) have called attention to the fact that anticoagulant substances of unknown nature may appear spontaneously in the circulating blood, giving rise to hemorrhagic diathesis. Recently Allen and his co-workers observed the occurrence of a "heparin-like" substance in the blood of animals following exposure to ionizing radiation (10, 11) and in the blood of patients treated with nitrogen mustards (14). These investigators also suggest that in other conditions associated with thrombocytopenia there may be an increased amount of heparin-like substance in the blood (12).

No very satisfactory method for detecting circulating anticoagulant has been available. Techniques which have been employed include the determination of the effect on the clotting time of normal blood of admixture with a small portion of the whole blood (3) or decalcified plasma (1) to be tested. The use of blood or plasma to which an anticoagulant has been added is undesirable because of the likelihood that this extraneous material itself may influence the outcome of the test. On the other hand the use of untreated whole blood is technically difficult because of its inherent tendency to clot, except in those unusual instances in which the blood is spontaneously incoagulable. In order to overcome these difficulties we have used native (undecalcified) platelet-free plasma for our anticoagulant assays.

## I. PREPARATION OF PLATELET-FREE PLASMA

Needles, syringes, test tubes and pipets to be used are treated with silicone[1] according to the technique described by Jaques and co-workers (11). A non-traumatic venipuncture is made with an 18 gauge silicone-treated needle. After several milliliters of blood have been with-

[1] General Electric Dri-Film 9987.

drawn into a syringe, the needle is left in place, the syringe disconnected and its contents discarded. This serves to rinse the needle free of any tissue juice which may have entered it during the venipuncture. A clean silicone-treated syringe is then attached to the needle, and 10 to 20 milliliters of blood are carefully withdrawn. The blood is placed in a silicone-treated test tube in an icebath. Immediately thereafter the tube is centrifuged at about 7000 RPM (about 6000 g) at 4°C. for 5 minutes to remove the blood cells and most of the platelets. The upper portion of the plasma is removed with a silicone-treated pipet and recentrifuged at about 12,000 to 14,000 RPM (about 17,500 to 22,000 g) for 10 minutes. The upper half of this plasma is then removed with a silicone-treated pipet and stored in a silicone-treated tube in an ice bath until required for use. Normal plasma obtained in this way may be kept for at least several hours at 4°C. without spontaneous coagulation. Plasmas containing a minute trace of anticoagulant are spontaneously incoagulable even when transferred to glass tubes at 37°C.

## II. ASSAY FOR ANTICOAGULANT

The presence of anticoagulant in the platelet-free plasma is demonstrated by observing its clot retarding effect on normal blood. Fifteen scrupulously clean Pyrex test tubes (13 mm. x 100 mm.) are mounted in a rack and to these are added physiological saline and platelet-free plasma as shown in Figure I.

With a silicone-treated needle and syringe sufficient blood is drawn from a normal individual to add one ml. portions quickly to each of the 15 tubes. In order to equalize the time factor the front row of tubes is filled from left to right, the middle row from right to left, and the rear row from left to right as indicated by the directions of the arrows in the diagram. A stopwatch is started at the time of the venipuncture when the blood first appears in the syringe. After the tubes have been filled, they are shaken uniformly to mix their contents, and the rack is placed in a water-bath at 37°C. The tubes of the front row are tilted periodically until a solid clot forms, after which the tubes of the middle and finally the rear row are similarly observed. The clotting time of each tube is noted and recorded.

Significant prolongation of the clotting times in the tubes containing

the platelet-free plasma is indicative of the presence of anticoagulant. Repeated observations have shown that variations in the quantity of the saline used in the control tubes from 0 to 0.5 ml. do not affect the clotting times. Normal platelet-free plasma may occasionally cause slight prolongation of the clotting time of normal blood. However, this rarely exceeds the clotting time of the saline controls by more than 7 minutes, whereas when anticoagulant is present the clotting time is usually greatly prolonged. When normal serum, fresh or stored, is

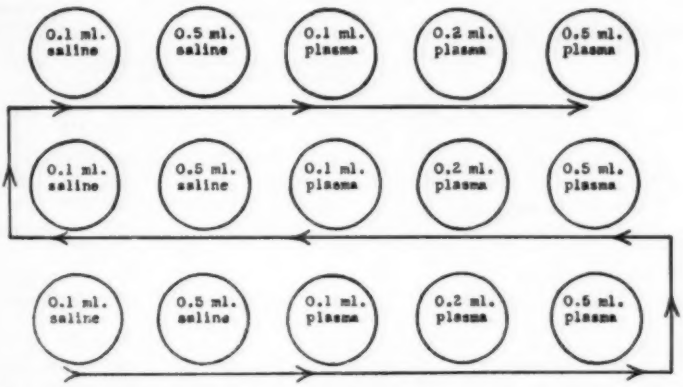


FIG. I. Arrangement of tubes for anticoagulant assay. The control tubes contain saline and the remaining tubes platelet-free plasma in the quantities indicated. In performing the test, one ml. portions of normal blood are added to each of the tubes in the order indicated by the arrows. The clotting time in each tube is recorded. The test is performed at 37°C.

used in place of the platelet-free plasma, there is no prolongation of the clotting time of normal blood. The circulating anticoagulants were always detectable by the use of platelet-free plasma, but in some patients not by the use of serum.

### III. APPRAISAL OF THE SENSITIVITY OF THE ASSAY

In order to determine the sensitivity of the assay procedure, tests on plasmas to which heparin[2] had been added *in vivo* and *in vitro* were undertaken. *In vitro* experiments were performed by the addition of

[2] Solution of Heparin, Sodium salt, Lederle Laboratories, Inc.

heparin to normal platelet-free plasmas, and *in vivo* experiments by obtaining native platelet-free plasma from normal individuals before and after the intravenous injection of heparin. Tables 1 and 2 show the results of these experiments.

TABLE 1

*A comparative study of the results of anticoagulant assays before and after the* in vitro *addition of varying amounts of heparin to normal platelet-free plasma*

| EXPERIMENT NUMBER | CONCENTRATION OF HEPARIN IN NORMAL PLATELET-FREE PLASMA | CLOTTING TIMES* OF 1.0 ML. PORTIONS OF NORMAL BLOOD CONTAINING: | | | | CLOTTING TIME OF PLATELET-FREE PLASMA IN GLASS TUBES |
|---|---|---|---|---|---|---|
| | | Saline 0.1 to 0.5 ml. | 0.1 ml. platelet-free plasma | 0.2 ml. platelet-free plasma | 0.5 ml. platelet-free plasma | |
| | *mgm. per ml.* | *min.* | *min.* | *min.* | *min.* | *min.* |
| I | None | 19 | 19 | 19 | 20 | 20 |
| | 0.01 | | 35 | 42 | >480 | No clots |
| II | None | 22 | 21 | 24 | 20 | 11 |
| | 0.01 | | 31 | 39 | >128 | No clots |
| III | None | 19 | 20 | 19 | 17 | 11 |
| | 0.001 | | 24 | 31 | 42 | No clots |
| IV | None | 15 | 14 | — | 12 | 11 |
| | 0.0005 | | 21 | 23 | 26 | No clots |
| V | None | 21 | 20 | 21 | 20 | — |
| | 0.0005 | | 20 | 22 | 29 | — |
| VI | None | 15 | 18 | 19 | 18 | — |
| | 0.00025 | | 22 | 22 | 29 | — |

* Clotting times were performed by a modified Lee-White method using 3 tubes at 37°C. Only the clotting time of the third tube is recorded in the tables for the sake of brevity.

From the results in Table 1 it is apparent that with plasma heparin concentrations of 0.001 mgm. per ml. and greater, this assay gives unequivocally positive results. Even with plasma heparin concentrations as low as 0.00025 mgm. per ml. there is a definite prolongation of the clotting time of normal blood, although this is within the limits sometimes seen with normal plasmas. Table 2 shows that following the intravenous injection of amounts of heparin too small to affect

TABLE 2

*Comparative Study of the Results of Anticoagulant Assays on Normal Individuals Before and After the Intravenous Injections of Varying Amounts of Heparin*

| EXPERIMENT NUMBER | | WHOLE BLOOD CLOTTING TIME OF SUBJECT | CLOTTING TIMES OF 1.0 ML. PORTIONS OF NORMAL BLOOD CONTAINING: | | | | | CLOTTING TIME OF PLATELET-FREE PLASMA IN GLASS TUBES |
|---|---|---|---|---|---|---|---|---|
| | | | Saline (0.1 to 0.5 ml.) | 0.1 ml. of platelet-free plasma | 0.2 ml. of platelet-free plasma | 0.5 ml. of platelet-free plasma | 0.5 ml. of platelet-free plasma + 0.1 ml. of toluidine blue* | |
| | | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* |
| I | Before heparin | 19 | 20 | 27 | — | 27 | — | 26 |
| | 16 minutes after 10 mgm. of heparin intravenously | 31 | | 39 | 43 | 61 | 27 | No clots |
| II | Before heparin | 20 | 22 | 24 | 25 | 23 | — | 54 |
| | 12 minutes after 7.5 mgm. of heparin intravenously | 19 | | 23 | 25 | 33 | 28 | No clots |
| III | Before heparin | 17 | 14 | 13 | 15 | 15 | — | 42 |
| | 21 minutes after 5 mgm. of heparin intravenously | 18 | | 19 | 20 | 20 | 23 | No clots |
| IV | Before heparin | 17 | 22 | 22 | 24 | 26 | — | 47 |
| | 16 minutes after 3 mgm. of heparin intravenously | 24 | | 26 | 28 | 30 | 35† | No clots |

* Toluidine Blue, Special Batch # 2, Serial E-1204, Abbott Laboratories. As a screening procedure only one concentration (0.5%) was used. One-tenth milliliter of this solution (or 0.5 mgm.) added to 1.0 ml. of normal blood containing 0.01 mgm. of heparin per milliliter was just sufficient to restore the clotting time to normal.

† See Discussion.

TABLE 3

*Results of Anticoagulant Assays on Forty-one Human Subjects Correlated with the Clotting Times of Whole Blood and of Platelet-free Plasma*

| SUBJECT | DIAGNOSIS | SUBJECT'S WHOLE BLOOD CLOTTING TIME WITH ADDED: | | ANTICOAGULANT ASSAY: Clotting time of 1.0 ml. portions of normal blood containing: | | | | | CLOTTING TIME OF PLATELET-FREE PLASMA IN GLASS TUBES AT 37°C. | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Subject's platelet-free plasma as follows: | | | | | |
| | | 0.1 ml. of saline | 0.1 ml. of toluidine blue (0.5%) | Saline (0.1 to 0.5 ml.) | 0.1 ml. | 0.2 ml. | 0.5 ml. | 0.5 ml. plus 0.1 ml. of toluidine blue (0.5%) | | |
| | | | | | Hemophilia | | | | | |
| | | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | |
| *1 | Hemophilia | 300 | — | 18 | 25 | 31 | 54 | — | No clots | — |
| 2 | Hemophilia | 390 | — | 10 | 10 | — | 8 | — | No clots | — |
| 3 | Hemophilia | 20 hrs. | — | 8 | 8 | 9 | 8 | — | No clots | — |
| 4 | Hemophilia | 33 | — | 16 | 19 | 19 | 18 | — | No clots | — |
| 5 | Hemophilia | 212 | — | 17 | 16 | 16 | 18 | 21 | No clots | — |
| 6 | Hemophilia | 240 | — | 22 | 20 | 20 | 22 | — | No clots | — |
| | | | | | Hemorrhagic diathesis due to circulating anticoagulant | | | | | |
| *7 | Undiagnosed disease with circulating anticoagulant | >24 hrs. | — | 13 | 39 | 75 | 75 | — | No clots | No shortening of whole blood clotting time with toluidine blue or protamine |
| *8 | Undiagnosed disease with circulating anticoagulant | 68 | — | 14 | 39 | 67 | — | — | No clots | No shortening of whole blood clotting time with toluidine blue or protamine |
| | | | | | Hemorrhagic diathesis with thrombocytopenia | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Hemorrhagic diathesis with thrombocytopenia** | | | | | | | | | |
| 9 | Acute leukemia (Infant. After 6.5 mgms. aminopterin) | 36 hrs. | — | 23 | 28 | 23 | 28 | — | No clots | Platelets 18,000. Bleeding time 4′. Positive tourniquet test |
| 10 | Acute leukemia (Before aminopterin) | 19 | 27 | 17 | 24 | 24 | 24 | 23 | 27 | Platelets 44,000. Bleeding time 11′ Negative tourniquet test |
| | (After 20.0 mgm. aminopterin | 9 | 15 | 18 | 21 | 21 | 19 | 17 | 11 | Platelets 22,000 |
| 11 | Acute leukemia (After 5.0 mgm. aminopterin) | 88 | 46 | 16 | 18 | 26 | 22 | 26 | No clots | Platelets 12,000 |
| | | | | 18 | 19 | — | 18 | 21 | — | |
| 12 | Acute leukemia | 7 | 18 | 12 | 11 | 14 | 12 | 14 | 18 | Platelets 26,000. Bleeding time 16′. Negative tourniquet test |
| 13 | Chronic lymphatic leukemia | 27 | — | 17 | — | 17 | 18 | 27 | 180 | Platelets 22,000. Bleeding time 11′. Positive tourniquet test |
| 14 | Thrombocytopenic purpura, primary | 15 | 25 | 15 | 16 | 16 | 15 | 21 | 27 | Platelets 30,000. Bleeding time 10′. Positive tourniquet test |
| 15 | Thrombocytopenic purpura, primary | 8 | 21 | 15 | 14 | 14 | 15 | 14 | 15 | Platelets 40,000. Bleeding time 10′. Positive tourniquet test |
| 16 | Refractory anemia with thrombocytopenia | 25 | 38 | 20 | 19 | 19 | 19 | 19 | 90 | Platelets 90,000. Bleeding time 90′. Positive tourniquet test |
| *17 | B. welchii septicemia | 52 | — | 18 | 27 | 28 | 32 | 30 | No clots | Platelets 70,000. Bleeding time 15′. Positive tourniquet test |
| 18 | Marchiafava-Micheli syndrome | 29 | — | 16 | — | — | 12 | 12 | No clots | Platelets 68,000. Bleeding time 1′. Positive tourniquet test |
| | **Hemorrhagic diathesis without thrombocytopenia** | | | | | | | | | |
| 19 | Non-thrombocytopenic purpura | 14 | — | 22 | 21 | 24 | 20 | 21 | 11 | Platelets & bleeding time normal Positive tourniquet test |
| 20 | Atypical hemorrhagic diathesis | 35 | — | 22 | 18 | 18 | 18 | — | No clots | Platelets, bleeding time, and tourniquet test normal |
| 21 | Multiple myeloma | 43 | — | 11 | 11 | 11 | 11 | — | 24 | Platelets & bleeding time normal. Positive tourniquet test |

TABLE 3—*Continued*

| SUBJECT | DIAGNOSIS | SUBJECT'S WHOLE BLOOD CLOTTING TIME WITH AIDED: | | ANTICOAGULANT ASSAY: Clotting time of 1.0 ml. portions of normal blood containing: | | | | | CLOTTING TIME OF PLATELET-FREE PLASMA IN GLASS TUBES AT 37°C. | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.1 ml. of saline | 0.1 ml. of toluidine blue (0.5%) | Saline (0.1 to 0.5 ml.) | Subjects platelet-free plasma as follows: 0.1 ml. | 0.2 ml. | 0.5 ml. | 0.5 ml. plus 0.1 ml. of toluidine blue (0.5%) | | |
| **Hemorrhagic diathesis with advanced liver disease** | | | | | | | | | | |
| | | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* | |
| *22 | Fatal acute hepatitis | 109 | — | 21 | 21 | 21 | 31 | — | No clots | Prothrombin 10% of normal. Antithrombic anticoagulant |
| *23 | Carcinoma of breast with | >24 hrs. | >24 hrs. | 17 | 32 | 37 | >14 hrs. | — | No clots | Prothrombin 5% of normal. |
| | carcinomatosis of liver | | | 20 | — | 28 | 135 | — | No clots | Antithrombic anticoagulant |
| †24 | Fatal acute hepatitis | 22 | — | 17 | 18 | 17 | 19 | 17 | 60 | Prothrombin <5% of normal. |
| **Hypoprothrombinemia due to dicumarol** | | | | | | | | | | |
| *25 | Coronary occlusion | 41 | — | 20 | 20 | 21 | 26 | — | No clots | Prothrombin 10% of normal |
| | | 26 | 26 | 19 | 22 | 26 | 34 | — | No clots | Prothrombin 10% of normal |
| | | 18 | — | 16 | 20 | 20 | 18 | 18 | 35 | Prothrombin 20% of normal |
| *26 | Fatal facial cellulitis | 55 | — | 22 | 28 | 29 | 32 | 22 | No clots | Prothrombin 30% of normal |
| | | 15 | 28 | 18 | 20 | 20 | 25 | 18 | No clots | Prothrombin 40% of normal |
| 27 | Coronary occlusion | 55 | 40 | 18 | 20 | 23 | 24 | 20 | No clots | Prothrombin <5% of normal |
| **Hodgkins disease treated with nitrogen mustards (No hemorrhagic diathesis)** | | | | | | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Hodgkins disease treated with nitrogen mustards (No hemorrhagic diathesis)** | | | | | | | | | | |
| 28 | Hodgkins disease | 22 | — | 16 | 17 | 17 | 20 | 18 | — | Before nitrogen mustards |
| | | 11 | 18 | 20 | 20 | 20 | 23 | 19 | 16 | Three days after treatment |
| | | 18 | — | 19 | 19 | 17 | 19 | 20 | 18 | Eleven days after treatment |
| | | 19 | — | 17 | 23 | 21 | 19 | 18 | 19 | Four weeks after treatment |
| 29 | Hodgkins disease | 16 | — | 15 | 16 | 18 | 18 | 18 | 52 | Before nitrogen mustards |
| | | 27 | — | 16 | 17 | 17 | 14 | 19 | 37 | Eight days after treatment |
| | | 26 | — | 20 | 17 | 18 | 21 | 18 | 180 | Two weeks after treatment |
| 30 | Hodgkins disease | 14 | 17 | 13 | 13 | 16 | 8 | 27 | 25 | During treatment |
| | | 20 | 20 | 14 | 16 | 13 | 19 | 14 | 54 | One week after treatment |
| | | 31 | — | 17 | — | 21 | 24 | 22 | 180 | Two weeks after treatment |
| **Subjects without evidence of hemorrhagic diathesis** | | | | | | | | | | |
| 31 | Diabetes mellitus | 23 | — | 19 | 19 | 19 | 20 | — | 20 | — |
| 32 | Diabetes mellitus | 32 | — | 21 | 23 | — | 28 | — | 140 | — |
| | | | | 21 | 20 | 21 | 20 | — | — | |
| | | | | 15 | 18 | 19 | 18 | — | — | |
| 33 | Post-op. appendectomy | 39 | — | 18 | 23 | 22 | 22 | — | 45 | — |
| 34 | Spastic clubfoot | 19 | — | 20 | 27 | — | 27 | — | 26 | — |
| 35 | Carcinoma of cervix | 16 | 20 | 18 | 20 | — | 20 | — | 18 | Irradiation sickness, moderate |
| 36 | Normal | 17 | — | 22 | 22 | 24 | 26 | — | 47 | — |
| 37 | Normal | 22 | — | 16 | 18 | 18 | 18 | 21 | 39 | — |
| 38 | Normal | 16 | — | 16 | 16 | 18 | 18 | 17 | 26 | — |
| 39 | Normal | 17 | — | 14 | 13 | 15 | 15 | — | 42 | — |
| 40 | Normal | 15 | — | 19 | 17 | 19 | 19 | — | 17 | — |
| 41 | Normal | 20 | — | 22 | 24 | 25 | 23 | — | 19 | — |

* Positive anticoagulant assay.

† This patient did not suffer from hemorrhagic diathesis, but is included for comparison with other patients suffering from liver disease.

appreciably the venous clotting time, this assay will still give suggestively positive results.

### IV. CLINICAL STUDIES

Forty-one subjects with or without evidence of hemorrhagic diathesis were studied using the technique described. These studies are summarized in Table 3.

Of the 41 subjects studied, eight showed evidence of circulating anticoagulants. In only one (#26) of these eight was the test reverted to normal by the addition of toluidine blue *in vitro*, suggesting the presence of a heparin-like substance. This patient had a severe facial cellulitis and hypoprothrombinemia due to dicumarol. In another patient with hypoprothrombinemia due to dicumarol (#25), the anticoagulant assay was positive on one occasion. Both of these patients had negative assays on subsequent tests. Of the six hemophilic patients studied, only one (#1) was found to have a circulating anticoagulant. Two patients (#7 and #8) previously reported (9) as having an undiagnosed disease with circulating anticoagulant showed markedly positive tests by our method. A positive test occurred in a patient (#17) with a bacillus welchii septicemia and thrombocytopenia following a septic abortion. In two of the three patients (#22, #23, #24) dying with severe liver disease, the anticoagulant assays were positive. We believe that these two patients (#23, #24) had antithrombic anticoagulants, because the clotting times of their plasmas on the addition of thrombin solution was prolonged markedly as compared to the clotting times of normal plasmas similarly treated.

In nine cases of thrombocytopenia (#9, 10, 11, 12, 13, 14, 15, 16, 18) the anticoagulant assays were negative. Three patients with leukemia (#9, 10, 11) showed negative assays after aminopterin therapy. Three patients with Hodgkins disease (#28, 29, 30) followed from two to four weeks after treatment with nitrogen-mustard[3] have had negative assays. Other subjects with and without hemorrhagic diathesis showed negative assays as listed in Table 3.

### DISCUSSION

A sensitive method for the detection of circulating anticoagulant by the use of native platelet-free plasma has been presented. In

[3] Methyl-bis (B-chloroethyl) amine hydrochloride (Merck), 0.1 mgm. per kilo body weight on 4 consecutive days.

every instance in which circulating anticoagulant was found, the platelet-free plasma was spontaneously incoagulable. This was true even when the clotting time of the whole blood was not markedly prolonged.

In controlled experiments with the addition *in vivo* and *in vitro* of amounts of heparin too small to affect greatly the whole blood clotting times, the detection of anticoagulant was nevertheless quite easy. It seems reasonable to assume that in any instance in which the whole blood clotting time is definitely prolonged due to the presence of anticoagulant, this assay would be unequivocally positive. Allen and his co-workers (12) suggest that a heparin-like anticoagulant may be associated with the hemorrhagic diathesis of certain cases of severe thrombocytopenia. In our small series of such cases we have been unable to detect the presence of any circulating anticoagulant even when the patients' whole blood clotting times were markedly prolonged and the patients were suffering from severe bleeding. If circulating anticoagulants occur in such patients as reported by Allen and his coworkers (12), their occurrence appears not to be a regular phenomenon.

In our experience, normal platelet-free plasma invariably clots in a relatively short time when placed in glass tubes at 37°C. This spontaneous coagulation of normal platelet-free plasma is completely inhibited by the addition of very minute amounts of heparin in concentrations too small to delay the clotting of whole blood. It seems quite reasonable to assume that the increased concentration of heparin required to inhibit or delay the coagulation of whole blood is roughly related to the number of platelets present. Allen and his associates (12) have described a test in which they determine the amount of protamine required to inactivate a given amount of heparin added *in vitro* to whole blood. These authors found that more protamine was necessary to restore the normal clotting time in thrombocytopenic blood than was required by normal controls, and suggested that in thrombocytopenia an increased amount of a heparin-like substance may be present in the blood. In view of our studies, it is quite apparent that the results of their tests would be influenced markedly by the platelet concentration. It seems to us that the increased susceptibility of thrombocytopenic blood to heparin, as reported by these authors, can be adequately explained by the deficiency of platelets without assuming the existence of an increased amount of a heparin-like substance circulating in the blood.

It can be seen from examination of Experiment III in Table 2 that, with a very low concentration of heparin, toluidine blue in the concentration used (0.5%) did not restore the clotting time of heparinized plasma to normal. This serves to reemphasize the well known fact that heparin inhibitors such as toluidine blue and protamine are in themselves anticoagulants. These substances correct a delayed clotting time due to heparin only when they are provided in concentrations just sufficient to inactivate the amount of heparin present. Further evidence of the anticoagulant action of these heparin inhibitors when present in excess is presented by the frequent prolongations of the clotting times when toluidine blue was added *in vitro* as shown in Table 3. Because of the reported value of the administration of anti-heparin compounds in hemorrhagic conditions associated with thrombocytopenia (12), two patients suffering from acute leukemia were given toluidine blue and protamine[4] intravenously in recommended doses. These substances failed to affect the hemorrhagic manifestations in any way. Furthermore, following the intravenous injection of from 50 to 200 mgm. of protamine the venous clotting times were greatly prolonged. In view of the anticoagulant effects of these drugs it appears quite possible that they may aggravate hemorrhage when given to patients who do not have hyperheparinemia.

It was not possible to ascertain the exact nature of the circulating anticoagulants in those cases in which they were found. Detailed studies on the anticoagulants of three patients (#1, 7, 8) have already been reported (9). One patient (#26) who had a severe facial cellulitis and hypoprothrombinemia due to dicumarol, showed the possible presence of a heparin-like substance on one occasion, but repeated studies did not confirm this, and we were unable to draw definite conclusions as to the nature of the anticoagulant. The nature of the anticoagulant in the patient (#17) with bacillus welchii septicemia following a septic abortion was not determined because the patient died before further studies could be carried out. One patient (#25) with hypoprothrombinemia due to dicumarol given therapeutically for a coronary occlusion failed to show the presence of the anticoagulant after dicumarol was discontinued and the prothrombin rose.

The patients with liver disease were of great interest. These three

[4] Protamine, Eli Lilly and Company.

patients (#22, 23, 24) were severely ill with ultimately fatal liver damage. The two (#22, 23) with positive anticoagulant assays had strong antithrombic anticoagulants in addition to a severe hypoprothrombinemia. As early as 1913, Whipple (15) noted that the plasma of a severely jaundiced patient with liver damage delayed the recalcified clotting time of normal dog plasma. The existence of increased *serum* antithrombic activity as an etiological factor in the hemorrhagic diathesis in obstructive jaundice has been postulated by other investigators (16, 17). However, their work is now subject to a more critical review in the light of more recent investigations on the hypoprothrombinemia associated with liver disease. Dyckerhoff and Marx (18) have presented experimental evidence for their opinion that an increase in *serum* antithrombin as well as hypoprothrombinemia may be concerned in the hemorrhagic diathesis in rabbits following ligation of the common bile duct. These authors stated that the question as to whether this was due to an increase in the normal *serum* antithrombin or to a specific inhibitory substance must be left to further investigation.

We are of the opinion that the antithrombins in our two cases were of a different nature than normal serum antithrombin in that they were truly anticoagulant. Clotting of these patients' *plasmas* by the addition of thrombin solution was markedly prolonged as compared to the clotting of normal plasmas. This was true even though the plasma fibrinogen levels were adequate. Therefore, we believe that these patients had active antithrombic anticoagulants in their plasmas rather than merely an increased thrombin-inactivating potency of their sera. Detailed studies on these and other cases of jaundice will be reported later.

In our experience, platelet-free plasmas from normal individuals invariably clot when placed in glass tubes at 37°C. If the platelet-free plasma clots, one can be certain that there is no anticoagulant present. When anticoagulant is demonstrated, its potency may be estimated by determining the smallest amount of platelet-free plasma necessary to prolong the clotting time of normal blood. One of our patients (#7) had an anticoagulant of such potency that 0.005 ml. of his platelet-free plasma produced an appreciable prolongation of the clotting time of 1 ml. of normal blood.

SUMMARY

1. A sensitive method for the detection of circulating anticoagulants is described. This method involves the use of platelet-free plasma to which no anticoagulant has been added *in vitro*.

2. The assay was found to be sensitive for concentrations of heparin too low to affect the whole blood clotting times appreciably.

3. Results obtained in 41 human subjects are presented and discussed.

BIBLIOGRAPHY

1. Lozner, E. L., Jolliffe, L. S., and Taylor, F. H. L.: Am. J. M. Sci. **199:** 318, 1940.
2. Lawrence, J. S. and Johnson, J. B.: Tr. Am. Climat. & Clin. Assn., 1941.
3. Craddock, C. G. and Lawrence, J. S.: Blood **2:** 505, 1947.
4. Munro, F. L. and Jones, H. W.: Am. J. M. Sci. **206:** 710, 1943.
5. Fantl, P. and Nance, M. H.: Med. J. Australia **2:** 125, 1946.
6. Chargaff, E. and West, R.: J. Biol. Chem. **166:** 189, 1946.
7. Castex, M. R.: Bull. de l'Academie de Medicine, Paris **130:** 596, 1946.
8. Quick, A. J. and Stefanini, M.: Proc. Soc. Exp. Biol. and Med. **67:** 111, 1948.
9. Conley, C. L., Rathbun, H. K., Morse, W. I., II, and Robinson, J. E., Jr.: Bull. Johns Hopkins Hosp. In Press.
10. Allen, J. G. and Jacobson, L. O.: Science **105:** 388, 1947.
11. Jaques, L. B., Fidlar, E., Feldsted, E. T., and Macdonald, A. G.: Canad. M. A. J. **55:** 26, 1946.
12. Allen, J. G., Bogardus, G., Jacobson, L. O., and Spurr, C. L.: Ann. Int. Med. **27:** 382, 1947.
13. Allen, J. G., Sanderson, M., Milham, M., Kirschon, A., and Jacobson, L. O.: J. Exp. Med. **87:** 71, 1948.
14. Smith, T. R., Jacobson, L. O., Spurr, C. L., Allen, J. G., and Block, M. H.: Science **107:** 474, 1948.
15. Whipple, G. H.: Arch. Int. Med. **12:** 637, 1913.
16. Hartmann, E.: Klin. Wchnschr. **6:** 1322, 1927.
17. Barlik, A.: Arch. f. Klin. Chir. **176:** 252, 1933.
18. Dyckerhoff, H. and Marx, R.: Biochem. Ztschr. **307:** 35, 1940–41.

# PHYSIOLOGICAL STUDIES IN CONGENITAL HEART DISEASE*

## VIII. The Physiological Findings in Two Patients with Complete Transposition of the Great Vessels

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Received for publication September 8, 1948

Previous papers from this laboratory have presented the results of physiological studies in patients with various congenital cardiac malformations. It is the purpose of this communication to report the results of physiological studies in two cases of complete transposition of the great vessels. In this congenital anomaly the aorta arises from the right ventricle, the pulmonary artery from the left. The great veins enter the heart normally (Fig. 1). The cases reported here were selected out of a series of five, since the results of the physiological studies could be correlated with clinical and postmortem findings.

### METHODS

The methods used in these studies have been described in detail in the first paper of this series (1). The Fick principle has been applied to determine the blood flow through various parts of the circulation. A definition of terms and a statement of the formulae as they apply to transposition of the great vessels follow.

Systemic flow is the volume of blood passing through the peripheral arterial vessels per minute.

$$\text{a. Systemic flow (ml. per. min.)} = \frac{\text{Oxygen uptake (ml. per. min.} \times 100)}{\left(\begin{matrix}\text{O}_2\text{ content of}\\ \text{peripheral ar-}\\ \text{terial blood}\\ \text{(vol. \%)}\end{matrix}\right) - \left(\begin{matrix}\text{O}_2\text{ content of}\\ \text{mixed venous}\\ \text{blood}\\ \text{(vol. \%)}\end{matrix}\right)}$$

Pulmonary artery blood flow is the blood flowing through the pulmonic valve into the pulmonary artery per minute.

* This study was supported by a grant from the Commonwealth Fund and the Carolyn Rose Strauss Foundation.

b. Pulmonary artery flow (ml. per. min.)

$$= \frac{\text{Oxygen uptake (ml. per. min.} \times 100)}{\begin{pmatrix}\text{O}_2\text{ content of}\\ \text{pul. vein blood}\\ \text{(vol. \%)}\end{pmatrix} - \begin{pmatrix}\text{O}_2\text{ content of}\\ \text{pul. art. blood}\\ \text{(vol. \%)}\end{pmatrix}}$$

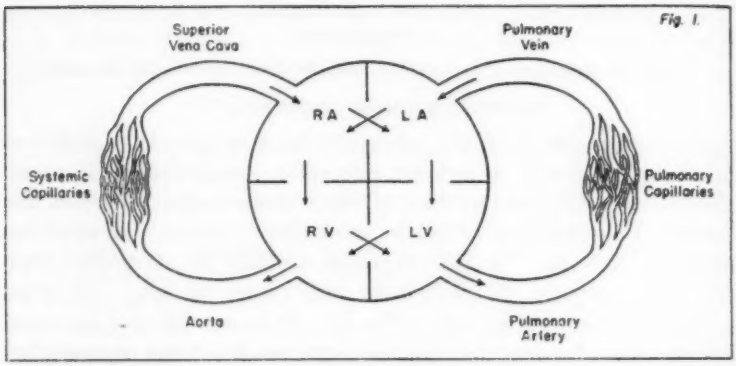


FIG. 1. Illustrates the anatomical arrangement present in congenital transposition of the great vessels. Auricular and ventricular septal defects have been included in this diagram to demonstrate possible means for admixture of blood between the two circulations.

The effective pulmonary blood flow is the volume of mixed venous blood which, after returning to the right auricle, eventually reaches the pulmonary capillaries.

$$\text{c. Effective pulmonary blood flow} = \frac{\text{Oxygen uptake (ml. per. min.} \times 100)}{\begin{pmatrix}\text{O}_2\text{ content of}\\ \text{pul. vein blood}\\ \text{(vol. \%)}\end{pmatrix} - \begin{pmatrix}\text{O}_2\text{ content of}\\ \text{mixed venous}\\ \text{blood (vol. \%)}\end{pmatrix}}$$

In calculating these flows, certain assumptions are made. When the oxygen content of pulmonary vein blood cannot be directly determined, a figure based on the assumption that pulmonary vein blood is 95 percent oxygenated is substituted in formulae b and c. In the presence of a ventricular septal defect, the oxygen content of right auricular blood is used to represent mixed venous blood in the determination of the systemic and the effective pulmonary blood flows. In

the presence of an auricular septal defect, however, the oxygen content of vena caval blood is assumed to be representative of mixed venous blood.

Blood oxygen values were determined from samples obtained during cardiac catheterization and by arterial puncture. Analysis of respiratory gases was carried out according to methods previously described (2).

*Case 1:* J. C. B. (Male, age 7; date of study, December 7, 1947).

The child was born at term following a normal pregnancy. At the age of two weeks when, because of persistent vomiting, he was taken to a pediatrician, a diagnosis was made of congenital heart disease with cyanosis. Although his development was slow and his exercise tolerance definitely limited, by the age of six years he was able to walk four or five blocks. During his first four years he always slept in the knee-chest position and often squatted to relieve dyspnea after exercise.

At the age of four months, the patient had bloody diarrhea; he also had some hematuria during his illness with chickenpox; and in the two years preceding admission he had had frequent episodes of "crops of petechiae" which disappeared spontaneously without specific therapy.

Physical examination revealed a small, cyanotic white boy of seven. He had clubbing of the fingers and toes. Tiny telangiectasis were present over the face, the volar surfaces of the forearms, and the legs. Capillary fragility tests were negative.

The pulse rate was 120 with regular rhythm. The blood pressure in the arms was 110/80. The heart percussed 6.5 cm. to the right of the midsternal line in the 5th interspace. A systolic thrill and murmur were most marked in the 2nd interspace to the left of the sternum, and the murmur could be heard over the left chest both anteriorly and posteriorly.

The lungs were clear to percussion and auscultation. There was no peripheral edema, and the liver and spleen were not enlarged. The remainder of the physical examination disclosed nothing remarkable.

The electrocardiogram showed right axis deviation and right ventricular hypertrophy. Routine laboratory studies revealed a hematocrit of 77.5 (Wintrobe), a hemoglobin of 23.5 grams (Sahli), and a red blood cell count of 9.5 million. On fluoroscopy the heart was slightly enlarged to the right; there was some evidence of a pulmonary conus, and the hilar shadows were thought to be too prominent for a typical tetralogy of Fallot. There was a left aortic arch. The pulmonary window was not clear.

Angiocardiography was performed with the following impression: "1. Complete transposition of the great vessels with the aorta arising entirely from the right ventricle. 2. Interauricular septal defect with the main overall shunt taking place from the right to the left auricle with re-opacification of the right ventricle

and aorta. The route of the venous blood to the lungs cannot be clearly ascertained."

*Physiological studies:* The standard exercise test was performed twice. On the first occasion the oxygen consumed per liter of ventilation rose from 27.0 cc. to 31.0 cc. A second test showed a fall from 34.2 cc. to 28.0 cc. Pertinent data obtained during cardiac catheterization are shown in Fig. 2. Significant findings include:

1. All four chambers of the heart were catheterized.

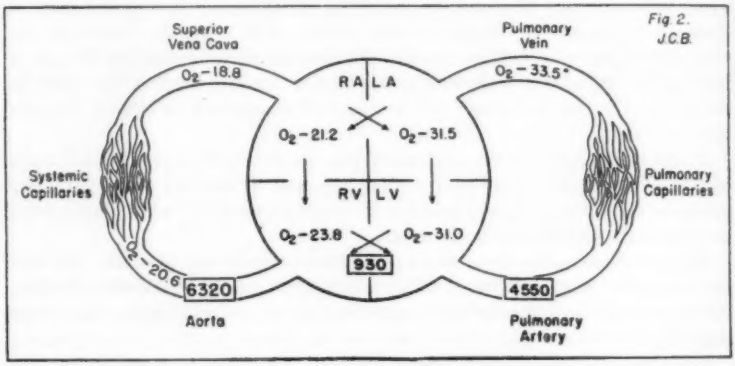


FIG. 2. Shows the findings in a patient (J. C. B.) with transposition of the great vessels. The increase in blood oxygen content from superior vena cava to right auricle demonstrates the presence of an auricular septal defect. The presence of a ventricular septal defect is shown by a rise in blood oxygen content from right auricle to right ventricle.

2. The oxygen content of the right auricular blood exceeded that of the superior vena cava by 2.4 volumes percent. The oxygen content of the right ventricular blood was 2.6 volumes percent higher than that of the right auricular blood.

3. The oxygen content of left auricular and left ventricular blood were within 0.5 volumes percent of one another. The oxygen saturation in these two chambers was about 93 percent.

4. The oxygen saturation of peripheral arterial blood was 59 percent.

5. Right ventricular and left ventricular pressures were 41/27 mm. Hg and 39/26 mm. Hg respectively.

*Comments:* The presence of both auricular and ventricular septal defects is suggested by the fact that all four chambers of the heart were intubated. Further evidence of the presence of these defects is that the oxygen content of right auricular blood is higher than that of the superior vena cava, and the oxygen content of right ventricular blood exceeds that of the right auricle. The oxygen content of peripheral arterial blood is lower than that of right ventricular blood. This may be due to the fact that the ventricular intracardiac samples were taken when the catheter tip lay close to the septal defect. The effective pulmonary flow was 930 cc/min/$M^2$.

On the 18th of December, 1947, a right thoracotomy was performed and the diagnosis of transposition of the great vessels was confirmed. The origin of the pulmonary artery was posterior to the aorta. The right pulmonary artery was divided and its distal end was anastomosed to the end of the right subclavian artery. An anastomosis between the proximal end of the right pulmonary artery and the side of the superior vena cava was performed. At the time of the operation the mean pulmonary artery pressure was 48 mm. Hg.

Immediately following the operation the patient's color seemed improved and the peripheral arterial oxygen saturation was 82 percent. He was digitalized with lanatoside c and was maintained on digifolin. About 12 hours postoperatively, however, the child began to respire with increasing difficulty; cyanosis developed and he became unconscious. Artificial respiration was of no avail.

Autopsy revealed the following pertinent findings: complete transposition of the great vessels, ventricular septal defect, patent foramen ovale, hypertrophy of the right and left ventricles.

The heart weighed 260 grams. Both ventricles were enlarged, especially the right. The right auricle was dilated and received the veins normally. The foramen ovale was covered by a membrane in which there were several openings. There was a high ventricular septal defect which was 8 mm. in diameter and partly covered by endocardium. The aorta arose from the right ventricle and its valve was normal. The left auricle was slightly dilated. The mitral valve was normal. The pulmonary artery arose from the left ventricle and coursed behind the ascending aorta. The pulmonic valve was normal, but the main trunk of the vessel was dilated and bulged at the point of division. The ductus arteriosus was closed.

*Case 2:* L. B. (Male, age 5 years; date of study, March 28, 1948).

The child was delivered at term after a normal pregnancy. He was cyanotic at birth and remained so. His development was moderately retarded and his exercise tolerance was markedly limited. He had had no episodes suggesting cardiac failure.

Physical examination disclosed a slight but fairly well nourished child of 5. The lips and mucous membranes were markedly cyanotic and the skin had a dusky

hue. The fingers and toes were clubbed and the nail beds were cyanotic. The left portion of the thoracic cage was larger in both diameters than the right. The lungs were clear to percussion and auscultation. The blood pressure in the arms was 90/65 mm. Hg. The left heart border was percussed 7.5 cm. from the midsternal line in the 5th interspace. The heart sounds were clearly heard, the rate and rhythm were normal, and no murmurs were heard. The liver was palpated 3 cm. below the costal margin and the spleen was felt.

Fluoroscopy showed the heart to be moderately enlarged in both the transverse and anteroposterior diameters. The pulmonary conus was slightly prominent. The markings at the right hilum were increased and pulsations were observed in the hilar vessels. A left aortic arch was demonstrated. There was no enlargement of the left auricle and both ventricles appeared prominent in the left anterior oblique view.

Electrocardiographic findings showed a right axis deviation with evidence in the unipolar precordial leads of right ventricular hypertrophy. The hematocrit was 97 (Wintrobe); the hemoglobin was 18.8 grams (Sahl), and the red cell count was 8.71 million.

Angiocardiography demonstrated passage of the contrast medium into both ventricles and filling of the aorta within two seconds after injection. Visualization of the pulmonary tree occurred later. The diagnosis was transposition of the great vessels with auricular septal defect.

*Physiological studies:* Pertinent data obtained from cardiac catheterization are shown in Fig. 3. The significant findings are as follows:

1. The oxygen content of the right ventricular blood was 2.7 volumes percent higher than that of the right auricular blood.

2. The aorta was catheterized through the right ventricle, and the oxygen content of aortic blood was 3.1 volumes percent higher than that of right ventricular blood.

3. The pressure in the right ventricle was 42/25 mm. Hg. A dampened pressure, 62/55, was recorded from the aorta.

*Comments:* The rise in oxygen content of the blood from the right auricle to the right ventricle indicates the presence of a ventricular septal defect allowing left to right admixture of blood. The observation that the oxygen content of the aortic blood is higher than that of right ventricular blood indicates the presence of a patent ductus arteriosis, shunting blood from the pulmonary artery into the aorta. The oxygen difference between the right ventricular and aortic blood also may be the result of the ventricular septal defect, through which a large volume of oxygenated blood is diverted into the right ventricle.

The pulmonary artery flow cannot be calculated since it was not possible to catheterize either the pulmonary artery or the left ventricle. The effective pulmonary flow was 500 cc/min/M².

On the 20th of April, 1948, a right thoracotomy was performed. The diagnosis of transposition of the great vessels was confirmed. The pulmonary artery was lying posterior to the aorta. The right pulmonary artery was divided and an anastomosis between the proximal end and the side of the superior vena cava was

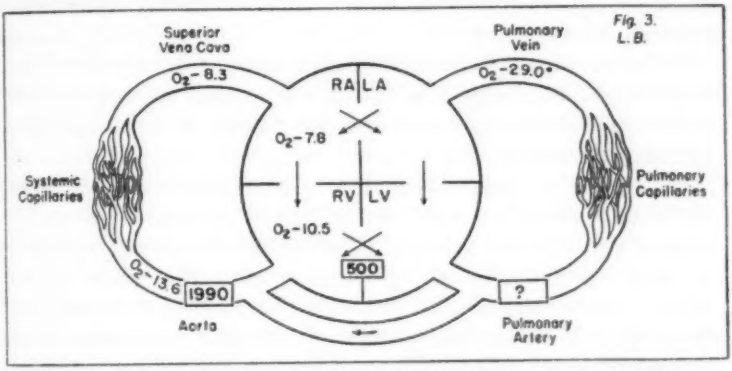


FIG. 3. Is a diagrammatic representation of the findings in a patient with transposition (L. B.). Although the blood oxygen contents of the superior vena cava and right auricle are essentially in agreement, post-mortem examination revealed a septal defect (patent foramen ovale). The rise in blood oxygen content from right auricle to right ventricle discloses the presence of a ventricular septal defect. The increased $O_2$ content of aortic blood compared to that of right ventricular blood suggests the presence of a patent ductus arteriosus. Shunt of blood in one direction through these defects must be compensated by concomitant admixture in the opposite direction.

performed. The distal end was ligated. Shortly after the chest wall was closed, respirations became poor and finally ceased.

Autopsy revealed the following findings: Transposition of the aorta and the pulmonary artery, patent foramen ovale, patent ductus arteriosus, ventricular septal defect, hypertrophy and dilatation of both ventricles, dilatation of the pulmonary artery and its branches, and arteriosclerosis of the pulmonary artery. The pulmonary artery was larger in circumference than the aorta. There was a high ventricular septal defect measuring 1.2 by 1 cm. The cusps of the mitral, tricuspid and aortic valves were normal; the foramen ovale and ductus arteriosus were both patent.

## DISCUSSION

The results reported in this paper demonstrate the difficulties encountered in the diagnosis of transposition of the great vessels by physiological methods alone.

The exercise test if of no particular value in the recognition of transposition. A fall in the rate of oxygen consumed per liter of ventilation from rest to exercise has been observed in the two cases reported in this paper. A decline in this ratio, however, also occurs in individuals with pulmonic stenosis.

Catheterization of the aorta through the right ventricle accomplished in patient L. B. is suggestive, but not definitive evidence, since the aorta is not infrequently intubated in tetralogy of Fallot through a high septal defect.

Recognition of this malformation by physiological tests is further hindered by difficulties encountered in the estimation of the pulmonary artery flow. Without advance knowledge of the abnormal origin of the great vessels, the blood $O_2$ content of right, rather than left ventricle, is erroneously used for the calculation of the pulmonary blood flow. Even if the correct diagnosis is suspected, calculation of the pulmonary artery flow is difficult since sampling of left ventricular blood is not easily accomplished. Once the diagnosis of transposition of the great vessels has been suggested by fluoroscopy or angiocardiography, however, physiological methods are of value in elucidating the intracardiac hemodynamics.

Some means must exist whereby oxygenated blood can reach the systemic circulation if transposition of the great vessels is to be compatible with extra-uterine life. Such interchange can take place through a patent ductus arteriosus, a patent foramen ovale, or a septal defect. In the first case described in this report (J. C. B.) both a patent foramen ovale and a ventricular septal defect were present. In the second case (L. B.) a patent ductus arteriosus, a patent foramen ovale, and a ventricular septal defect were found.

In cases of septal defect without transposition, the overall intracardiac shunt is unidirectional and may be computed as the difference between the volume of the systemic and the pulmonary flows. Thus, when the systemic blood flow exceeds that through the pulmonary artery, the overall direction of the shunt is from right to left.

Conversely, if the pulmonary artery flow exceeds the systemic flow, the overall direction of the shunt is from left to right. Equilibrium of the blood volumes between the pulmonary and the systemic circulation is maintained in these cases since the quantity of the overall shunt is returned to the side of the heart from which it was originally diverted.

On the other hand, in transposition of the great vessels, a unidirectional shunt cannot exist continuously as it would lead to progressive depletion of the circulating blood volume in either the pulmonary or the systemic circulation. The crucial hemodynamic alteration in transposition of the great vessels is that the volume of blood, shunted from pulmonary to systemic circulation or vice versa, cannot return to that side of the heart from which it was diverted unless there is an equivalent, opposite shunt. One must postulate, therefore, that in this condition equal volumes of blood are reciprocally shunted from one circulation to the other.

Since the effective pulmonary blood flow is the volume of venous blood which, after its return to the right auricle, is eventually aerated in the lung, it must be represented by the right to the left shunt. According to the considerations presented above, an equivalent amount of blood also must be shunted from left to right. Thus, a computation of the effective flow by methods outlined above also reveals the amount of right to left shunt and the corresponding left to right shunt.

Although, eventually, the shunt must be equal in both directions, the possibility exists that over a short period of time the shunt may be predominantly toward one side. Several observations bear out this possibility. First, it has been shown that the peripheral oxygen saturation is a direct function of the ratio of the effective pulmonary blood flow to the systemic circulation (2). However, in the first case (J. C. B.) the effective pulmonary flow represents 14 percent of the systemic circulation, and the peripheral arterial oxygen saturation is 59 percent. In the second case (L. B.), the effective flow is 25 per cent of the systemic flow, but the peripheral arterial saturation is only 44 percent. Since mixed venous and peripheral arterial blood samples were not collected simultaneously, a change in the direction of the intracardiac shunt between sampling might explain this discrepancy. A second observation which may be construed as evidence for the possibility that the direction of the intracardiac shunt may shift periodically

is the fluoroscopic finding of Taussig that the auricles may show a rhythmic increase and decrease in size, independent of respiratory changes (3). It becomes apparent, therefore, that measurement of the effective flow, or in other words, the right to left shunt, is valid only for a given time.

It might be expected that intracardiac pressures might reflect these changes in volume or direction of the intracardiac shunt. Pressures obtained in the first patient (J. C. B.) were 41/27 from the right ventricle and 39/26 from the left, and the contours of the pressure curve were similar. No variations in amplitude or contour occurred which could be interpreted as significant. Adequate pressure tracings were recorded from the right auricle. Again, no intermittent change was noted. Consequently, while the possibility exists that the volume and direction of the intracardiac shunt may vary over short periods of time, no definite physiological evidence for this phenomenon has been observed.

## SUMMARY

Physiological studies have been performed on two patients with complete transposition of the great vessels. The results obtained were correlated with the clinical and postmortem findings.

A discussion of the intracardiac hemodynamics has been presented.

## BIBLIOGRAPHY

1. Bing, R. J., Vandam, L. D., and Gray, F. D., Jr.: Physiological Studies in Congenital Heart Disease. I. Procedures. Bull. Johns Hopkins Hospital, **80:** 107, 1947.
2. Bing, R. J., Vandam, L. D., and Gray, F. D., Jr.: Physiological Studies in Congenital Heart Disease. II. Results of Preoperative Studies in Patients with Tetralogy of Fallot. Bull. Johns Hopkins Hospital, **80:** 121, 1947.
3. Taussig, H. B.: Congenital Malformations of the Heart. The Commonwealth Fund. 1947.

# MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

## HURD HALL, JOHNS HOPKINS HOSPITAL

## MONDAY, DECEMBER 13, 1948—8:15 P.M.

*Dr. Harvey:* The first paper on the program this evening is on "The Clinical Studies of Two Polymethylene."

I am very sorry to say that in printing up the program the name of one of the collaborators was inadvertently omitted—the name of Dr. Holaday of the Department of Pharmacology.

The paper will be given by Dr. Grob.

*Some Preliminary Observations on The Neuromuscular and Ganglionic Blocking Action in Man of bis-Trimethylammonium Decane and Pentane Dirodide.* DAVID GROB, A. MCGEHEE HARVEY AND DUNCAN A. HOLADAY.

d-Tubocurarine chloride blocks conduction across the neuromuscular junction, and its ability to thereby produce relaxation of skeletal muscle has been employed during surgical anesthesia and convulsive shock therapy. Tetraethylammonium chloride blocks conduction across autonomic ganglia, and has been employed to produce vasodilatation in patients with peripheral vascular disease, and in the study of the various types of hypertension. Both of these compounds have some undesirable features. d-Tubocurarine, when administered in amounts sufficient to produce relaxation of the muscles of the abdomen, trunk and extremities also produces marked weakness of the muscles of swallowing and often of respiration. In addition, d-tubocurarine occasionally causes a fall in blood pressure and sometimes bronchoconstriction, probably due to the release of a histamine-like substance from the tissues. Tetraethylammonium also has been somewhat limited in its efficacy, as a result of its short duration of action and some unexplained variations in its effects.

In a search for new blocking agents Paton and Zaimis have studied the pharmacologic properties in animals of the bis-trimethylammonium series of compounds in which the only variation is the length of the polymethylene chain connecting the two quaternary ammonium groups. They synthesized the compounds containing 2 through 12, and 18, carbon atoms in this chain, and referred to each compound by the number of carbon atoms in the chain. Their animal work showed that the C-10 compound is a neuromuscular blocking agent which appears to have relatively less effect than d-tubocurarine on the muscles of respiration, and less histaminic effect. The C-5 compound was found to be a ganglionic blocking agent, similar in its effects to tetraethylammonium, but more potent and longer acting.

The administration of the C-10 and C-5 compounds to human subjects has indicated that they will probably be clinically useful. The intravenous administration of 3 mg. of C-10 to normal human subjects resulted in weakness of the extraocular, facial, jaw, neck, and trunk muscles and almost complete paralysis of the muscles

of the extremities. This weakness could be maintained by the intravenous injection of 0.5 mg. of C-10 at five minute intervals. When sufficient C-10 had been administered to cause almost complete paralysis of the muscles of the extremities there was relatively slight weakness of the muscles of swallowing, speech and respiration. In contrast, when sufficient d-tubocurarine was administered to produce only moderate weakness of the arm muscles, and slight weakness of the legs, there was marked impairment of swallowing and speech and slightly greater impairment of respiration than had been encountered at a greater degree of peripheral relaxation produced by C-10. The effective dose of C-10 was only one-third that of d-tubocurarine on either a weight or molar basis, and its action was slightly less prolonged. C-10 produced no fall in blood pressure or other manifestation suggesting the release of a histamine-like substance. C-10, like d-tubocurarine, produced a progressive decline in the muscle action potentials initiated by supramaximal nerve stimuli, but the mechanism of action of the two compounds does not appear to be the same.

The blocking action of C-5 on autonomic ganglia has been studied by observing the effect of this compound on the blood pressure. The administration of 5 to 12 mg. of C-5 intravenously usually resulted in a postural hypotension in normal subjects and some hypertensive patients, and a prompt fall in blood pressure in some hypertensive patients. Patients with fixed hypertension had the least response to C-5 or tetraethylammonium. The effective dose of C-5 was only one-tenth to one-twentieth that of tetraethylammonium, and its action was more prolonged.

*Dr. Harvey:* Dr. Grob's paper is now open for discussion. Dr. Holaday, would you like to make any comments?

*Dr. Holaday:* The studies that have been made with this compound so far have not been carried to the degree that I think we should make any broad statements about its future value, but it seems to give sufficient promise that the anesthesiologists may find it a worthwhile substitute, at least in certain cases, for curare preparations that are now being used, inasmuch as it does not seem to have the histamine-like releasing property that curare preparations seem to have. Other than that, in the field of anesthesia at least, its action seems to be very similar to that of curare, with perhaps the exception that it spares the muscles of respiration and muscles innervated by cranial nerves to a somewhat greater extent than does d-tubocurarine. This may detract somewhat from its value in anesthesia, inasmuch as curare is not infrequently used in troublesome laryngospasms that may arise in the course of pentothal or cyclopropane induction. If the property of this drug that has been described in the preliminary studies—that it does favor the muscles of respiration somewhat more than does the curare, as compared to its paralyzing effect on the muscles of the extremities—it also might have slight disadvantages as compared with curare, inasmuch as the abdominal muscles are the muscles that give the surgeon primary concern as far as relaxation is concerned. As these muscles are innervated by the intercostal nerves, those supplying muscles of respiration, one may

have to encroach somewhat more upon the respiratory mechanism in producing adequate relaxation for anesthesia than would be necessary in the case of the use of this drug C-10 in combatting the bone-breaking convulsions of shock therapy as employed in the treatment of certain psychoses.

*Dr. Harvey:* These drugs are of more than usual interest as far as basic physiological mechanisms are concerned in that the C-10 compound is not antagonized at all by neostigmine, but curiously enough, if the animal has received d-tubocurarine prior to administration of the C-10, the blocking action is decreased.

One of our main interests in this whole problem is that pain pathways have synapses too, and, perhaps, if we understand more about these drugs that affect synapses in various regions—and here we have two that are very closely related as far as the active chemical group is concerned, each of which has specific action on different synapses—as we learn more about this action it may lead us to a drug which is a specific blocker of pain.

*Dr. Rich:* May I ask one question? I understood Dr. Grob to say that the injection of d-tubocurarine produces wheals and acidity. Can this be neutralized or prevented by benzamine? I would like to ask also whether increased gastric acidity accompanies the injection.

*Dr. Grob:* We have not studied the effect of antihistaminic agents on the increased secretion of gastric acid induced by d-tubocurarine. As you have indicated, the histamine-induced secretion of gastric acid is said to be not inhibited by antihistaminic agents, so that a study of this kind might throw added light on the resemblance between the histamine-like substance liberated after d-tubocurarine itself. There is no evidence that this substance is identical with histamine, but in the studies performed thus far it has had similar effects.

*Dr. Wilkinson:* Is there any correlation between the response that hypertensive patients have with these drugs and the benefit they may expect to gain from sympathectomy?

*Dr. Grob:* We haven't given a sufficient number of patients C-5 to enable us to make such a comparative study. Tetraethylammonium has been given to a large number of patients by other investigators prior to sympathectomy and there has been conflicting evidence as to its value in predicting the success of that operation. Some investigators say that they cannot predict the value of sympathectomy by the response to tetraethylammonium. Others, who appear to have been more careful in studying the effect of tetraethylammonium on the blood pressure in the erect, as well as the supine position, feel that there is frequently a correlation between the effects of tetraethylammonium and of sympathectomy on the blood pressure.

*Dr. Harvey:* Dr. Thomas, would you like to make any comments?

*Dr. Thomas:* I would certainly like to point out that the results of the sympathectomy operation depend on the clinical condition of the patient.

*Dr. Harvey:* The next two papers form a group and I think we will hear both of them before the discussion is opened. The first of these will be given by Dr. John Seed and is on "The Mode of Action of 933-F Benzodioxine."

*On the Mechanism of Action of 933F.* JOHN C. SEED AND EVAN CALKINS.

Piperidinomethyl-3-benzodioxane (933F) blocks temporarily the vasoconstrictor action of epinephrine but does not block the vasodilator action. Hence, after 933F epinephrine causes a fall in blood pressure, the fall being due primarily to vasodilation in striated muscle. Norepinephrine has no vasodilator action, so that its pressor action is blocked but not reversed by 933F. Most effects of epinephrine, such as those on the intestine, on the isolated vascular bed of nose and spleen, and on the blood sugar are blocked but not reversed. The accelerating effect of epinephrine on the denervated mammalian heart is not blocked. 933F blocks the action of sympathin but not the effects of sympathetic stimulation. 933F blocks the action of other pressor amines to a varying degree.

Compounds related to 933F which block the pressor action of epinephrine have in common a benzene ring linked to an oxygen which in turn is linked to an ethyl amine. These synthetic compounds have a common spatial configuration with the ergotoxine and yohimbine alkaloids. This structure consists of an oxygen linked to two carbons and separated by a distance equivalent to two carbon atoms (about 4.0–4.5 Angstroms) from a trivalent nitrogen. In the ergotoxine alkaloids the configuration exists in the amide side chain. Alterations in the ergotoxine molecule which affect this configuration abolish epinephrine-blocking activity whereas alterations in the lysergic acid part of the molecule do not. The change from yohimbine to yohimbic acid affects this configuration and also abolishes epinephrine-blocking activity.

It is postulated that compounds with this configuration compete with epinephrine for an "active surface." This "active surface" contains receptors for the alcoholic hydroxyl group and for the amine group in epinephrine. The competition between epinephrine and the blocking compounds is for this alcohol-amine receptor. It is proposed that epinephrine exerts its action by first combining with the "active surface" and then exerting an effect. If the "active surface" is occupied, then epinephrine cannot combine with it and exert an effect. It is postulated further that dibenzyl-β-chlorethylamine (Dibenamine), which irreversibly blocks the pressor action of epinephrine, combines with or near this same alcohol-amine receptor. If the receptor is occupied already by another blocking compound, such as 933F, then Dibenamine should be unable to combine with the receptor. By testing whether the epinephrine blockade after an injection of 933F followed by Dibenamine is reversible or irreversible one can determine whether 933F or Dibenamine combined with the receptor.

5 mg/K of 933F completely prevents 10 mg/K of Dibenamine from blocking the pressor action of epinephrine in the dog anesthetized with sodium pentobarbital.

*Dr. Harvey:* The final paper is on "Clinical Experience with 933-F in Hypertensive Patients." The paper will be given by Dr. George Dana.

*Clinical Experience with Benzodioxane.* Dr. George W. Dana and Dr. Evan Calkins.

Clinical experience with Benzodioxane, 933F, in 67 hypertensive patients is reported. In each adult case, 20 mgms. of the drug in saline, was given intravenously during a two minute period. In no instance was there any lasting discomfort. The usual coincident signs of the test were tachycardia, flush, and sighing respirations.

Sixty-five cases gave what was interpreted as a negative Benzodioxane test; namely, a variable rise in blood pressure. In the majority of these sixty five cases, the referring physician suspected that the cause for the hypertension might be a chromaffin tumor.

One patient is described with sustained hypertension and symptoms of nausea, and bouts of perspiration, and weakness, whose blood pressure fell from 170/120 to 92/60 in three minutes following injection of 933F. Operation revealed a phaeochromocytoma of the left adrenal.

In a second patient, aged six, suffering with sustained hypertension, the blood pressure fell from 200/145 to 160/100 in two minutes, following injection of Benzodioxane. At operation a large cell neuroblastoma was encountered, incorporating the left adrenal, and enclosing the upper pole of the left kidney. This test was interpreted as a false positive, and is yet to be adequately explained.

The greatest potential value to the clinician of 933F appears to be in determining whether sustained hypertension is due to a phaeochromocytoma.

Further experience with this drug will undoubtedly define its diagnostic usefulness more accurately, but even now the Benzodioxane test can be included appropriately in the survey of hypertensive patients.

*Dr. Harvey:* These two papers are now open for discussion. Dr. Howard, would you like to make any comments?

*Dr. Howard:* Dr. Pincoffs, here, was the first to diagnose one of these cases. Perhaps he would start the discussion.

*Dr. Pincoffs:* We have had a limited experience with the benzodioxane test. In a series of approximately 44 cases, we have not encountered a case of pheochromocytoma. I feel, incidently, that the author of this interesting paper was fortunate to have discovered one of these rare tumors in his series since the incidence is

certainly not high enough to have made this probable. Of course the author's cases were selected from those who clinically suggested pheochromocytoma.

We experienced the same difficulty that has been mentioned,—that of interpreting the meaning of the rise in blood pressure following the introduction intravenously of benzodioxane. We assumed that perhaps this rise in pressure was of psychic origin. It was found that when the procedure of the test was altered by establishing first a slow intravenous infusion of normal saline with the apparatus out of sight behind the patient and then introducing the benzodioxane by injection into the rubber tubing of the infusion set without the patient's knowledge, the rise in blood pressure was minimized. We did not infer however that in some instances these pressure rises may not be due to drug action. We did feel that in some there was a distinct psychic component.

*Dr. Harvey:* With such a pharmacological evening as this, I am sure Dr. Blanchard is anxious to add to this discussion.

*Dr. Blanchard:* Just as a matter of historical interest I might point out that the technic used by Dr. Seed, namely, calculation of the distance between two or more atoms of a drug, is an aspect of the theory of drug activity employed by chemists for a number of years past. Fourneau actually employed this concept in developing drugs of the 993-F series, only what he was trying to design was an antimalarial drug. These drugs turned out to exhibit the activity that we have heard about this evening, after remaining on the shelves for some time.

*Dr. Harvey:* Are there any further questions or comments? The meeting is closed.